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# BULLETIN OF THE AGRICULTURAL CHEMICAL SOCIETY OF JAPAN

## CONTENTS

1. K. Goto, I. Yamamoto and S. Matsumoto: On the Series Relation of Bimolecular Alkaloids of Sinomenine Type and the Cause of Their Existence. .............................. 1
2. H. Katagiri and K. Imai: Studies on Lactic Acid Fermentation. Part 1. ................. 9
3. H. Katagiri and K. Imai: Studies on Lactic Acid Fermentation. Part 2. ................. 15
4. T. Yamamoto: Report I. Studies on the $\alpha$-Amylase Destroying Enzyme. Part 1. ...... 22
5. K. Maekawa und M. Kushibe: Über die Oxydation des Lysozyms mit Perjodat. ......... 28
6. Y. Kuroiwa and Y. Horie: Studies on Nucleic Acid and Related Substances. Part 1. ...... 35
7. M. Matsui and S. Kitamura: Studies on Chrysanthemic Acid. Part 1. ..................... 42
8. I. Yamasaki, S. Ueda and S. Hayashida: A Microscopic Study on the Inner Structure of Starch Granules. ......................................................................................... 48
9. K. Arima, K. Komagata, S. Sugiyama, M. Kazama and K. Yano: Metabolism of Aromatic Compounds by Microbes. Part 4. ............................................................ 51
10. K. Arima, K. Komagata, M. Kazama, S. Sugiyama and K. Yano: Metabolism of Aromatic Compounds by Microbes. Part 5. ............................................................ 61
11. S. Kawamura, T. Koboyashi, M. Ôshima and M. Mino: Studies on the Carbohydrates of Soybeans. V. ............................................................................................ 69
12. K. Nishida, A. Kobayashi and T. Nagahama: Studies on Cycasin, a New Toxic Glycoside, of *Cycas revoluta* Thunb. ........................................................................ 77
13. A. Kawarada, N. Takahashi, H. Kitamura, Y. Seta, M. Takai and S. Tamura: Biochemical Studies on "*Bakanae*" Fungus. Part 33. ............................................ 84
14. K. Miyao: Studies on Fungisporin. Part 2. ................................................................ 86

**Communications.**

15. M. Abe, S. Yamatodani, T. Yamano and M. Kusumoto: On a New Water-Soluble Ergot Alkaloid, Triseclavine. .............................................................................. 92
16. S. Yamatodani and M. Abe: The Structure of Ergot Alkaloid, Elymoclavine ............... 94

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1955

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# CONTENTS OF VOLUME 19, 1955

## No. 1.

K. GOTO, I. YATAMOTO and S. MATSUMOTO: On the Series Relation of Bimolecular Alkaloids of Sinomenine Type and the Cause of Their Existence. ... 1

H. KATAGIRI and K. IMAI: Studies on Lactic Acid Fermentation. Part 1. ... 9

H. KATAGIRI and K. IMAI: Studies on Lactic Acid Fermentation. Part 2. ... 15

T. YAMAMOTO: Report I. Studies on the $\alpha$-Amylase Destroying Enzyme. Part 1. ... 22

K. MAEKAWA und M. KUSHIBE: Über die Oxydation des Lysozymes mit Perjodat. ... 28

Y. KUROIWA and Y. HORIE: Studies on Nucleic Acid and Related Substances. Part 1.... 35

M. MATSUI and S. KITAMURA: Studies on Chrysanthemic Acid. Part 1. ... 42

I. YAMASAKI, S. UEDA and S. HAYASHIDA: A Microscopic Study on the Inner Structure of Starch Granules. ... 48

K. ARIMA, K. KOMAGATA, S. SUGIYAMA, M. KAZAMA and K. YANO: Metabolism of Aromatic Compounds by Microbes. Part 4. ... 51

K. ARIMA, K. KOMAGATA, M. KAZAMA, S. SUGIYAMA and K. YANO: Metabolism of Aromatic Compounds by Microbes. Part 5. ... 61

S. KAWAMURA, T. KOBAYASAI, M. ÔSHIMA and M. MINO: Studies on the Carbohydrates of Sobybeans. V ... 69

K. NISHIDA, A. KOBAYASHI and T. NAGAHAMA: Studies on Cycasin, a New Toxic Glycoside, of *Cycas revoluta* Thumb. ... 77

A. KAWARADA, N. TAKAHASHI, H. KITAMURA, Y. SETA, M. TAKAI and S. TAMURA: Biochemical Studies on "*Bakanae*" Fungus. Part 33. ... 84

K. MIYAO: Studies on Fungisporin. Part 2. ... 86

**Communications.**

M. ABE, S. YAMATODANI, T. YAMANO and M. KUSUMOTO: On a New Water-Soluble Ergot Alkaloid, Triseclavine. ... 92

S. YAMATODANI and M. ABE: The Structure of Ergot Alkaloid, Elymoclavine. ... 94

## No. 2.

K. AIDA: Studies on Oxidative Fermentation. Part XIII. ... 97

T. NAKABAYASHI: Isolation of Astragalin and Isoquercitrin from Bracken, *Pteridium aquilinum* ... 104

K. GOTO, and I. YAMAMOTO: Studies on Shinomenine. Part LXVIII ... 110

T. YAMAMOTO: Studies on Sensitive Groups of Crystallin Bacterial $\alpha$-Amylase. ... 121

T. MUTO and Y. SAHASHI: Biochemical Studies on Vitamin $B_{12}$. Part X. ... 129

I. ŌNISHI and K. YAMASAKI: Studies on the Essential Oils of Tobacco Leaves. Part I. ... 137

I. ŌNISHI and K. YAMAMOTO: Studies on the Essential Oils of Tobacco Leaves. Part II. ... 143

I. ŌNISHI and M. NAGASAWA: Studies on the Essential Oils of Tobacco Learves. Part III. ... 148

K. Michi and H. Nonaka: Enzymatic Resolution of Racemic Amino Acid. Part III. 153

**Note.**

M. Matsui and M. Miyano: Studies on Chrysanthemic Acid. Part II. .................... 159

**Communication.**

M. Abe and S. Yamatodani: On a New Water-Soluble Ergot Alkaloid, Molliclavine... 161

## No. 3.

T. Kobayashi: Studies on Isoamylase. Part VIII................................................ 163
A. Baba and H. Kojima: Absence of of $\beta$-Glucosidase-Labile Linkage in Amylose. ... 167
K. Nishida, A. Kobayashi and T. Nagahama: Studies on Cycasin, a New Toxic Glycoside, of *Cycas revoluta* Thumb. Part II................................................ 172
T. Shimomura and Y. Nakamura: Biochemical Studies of *Piricularia oryzae.* Part III................................................ 178
K. Fukunaga, T. Misato, I. Ishii and M. Asakawa: Blasticidin, A New Anti-Phytopathogenic Fungal Substance. Part I. ................................................ 181
Y. Obata, S. Sakamura and M. Koshika: Isolation and Identification of $\alpha$-Spinasterol and $\alpha$-Spinasterol D-Glucoside from Sugar Beet Pulp. ........................................ 189
Y. Inouye, Y. Takeshita and M. Ohno: Studies on Synthetic Pyrethroids. Part V.... 193
H. Mitsuda and K. Yasumatsu: Crystallization of Animal Catalase and Studies on its Optimum Temperature. ................................................ 200
H. Mitsuda and K. Yasumatsu: Studies on Plant Catalase. .................................. 208
Y. Inouye and M. Noda: Separation and Identification of Fatty Acids. Part XVII.. 214

## No. 4.

T. Andō and Y. Ikeda: Paperelectrophoretic Studies on the Diastatic Enzymes of Molds. Part I................................................ 221
T. Andō: Paperelectrophoretic Studies on the Diastatic Enzymes of Molds. Part II.. 228
K. Izaki, H. Takahkshi and K. Sakaguchi: Studies on the Metabolism of D-Amino Acid in Microorganisms. Part I. ................................................ 233
H. Iwamoto and G. Yonekawa and T. Asai: Fat Synthesis in Unicellular Algae. Part I................................................ 240
H. Iwamoto and N. Nagahashi: Fat Synthesis in Unicellular Algae. Part II.......... 247
Y. Fujimoto and J. Sakamoto: The Influence of Magnesium Deficiency on the Growth of Chlorella. ................................................ 253
S. Maruno: Islanditoxin, a Toxic Metabolite Produced by *Penicillium islandicum.* Sopp. Part I. ................................................ 258
S. Maruno, K. Miyao and A. Matsuyama: Islanditoxin, a Toxic Metabolite Produced by *Penicillum islandicum.* Sopp. Part II. ................................................ 262
N. Takahashi, H. Kitamura, A. Kawarada, Y. Sema, M. Takai, S. Tamura and Y. Sumiki: Biochemical Studies on "Bakanae" Fungus. Part XXXIV. ........... 267
A. Kawarada, H. Kitamura, Y. Seta, N. Takahashi, M. Takai, S. Tamura and Y. Sumiki: Biochemical Studies on "Bakanae" Fungus. Part XXXV............... 278
Y. Sahashi, T. Muto and S. Asai: Biochemical Studies on Vitamin $B_{12}$. Part XI.... 282

**Note.**

M. Yamada and S. Masai: Studies on the Detection of Pyruvic Acid. .................... 289

The publication of the Bulletin of the Agricultural Chemical Society of Japan was suspended for more than ten years on account of the war. Now, the financial condition of the society having been improved to some degree, we are very glad to resume its publication as a quaternary journal. As the former series ended with Vol. 18 (1942), the new series will begin with Vol. 19 (1955).

Besides, we continue to publish our monthly journal in Japanese as before. The articles appearing in the latter are provided with short resume in English and we hope that they are also accessible to the foreign readers.

It is our sincere hope that these two publications will be availed by our foreign colleagues, who are interested in the research works, carried on in Japan, in the field of agricultural chemistry and its concerning problems.

January, 1955

Y. Okuda

Yuzuru OKUDA
President of the Agricultural
Chemical Society of Japan.

# 1. *On the Series Relation of Bimolecular Alkaloids of Sinomenine Type and the Cause of Their Existence.*[1]

By Kakuji GOTO, Izuru YAMAMOTO and Shiro MATSUMOTO

Alkaloids of sinomenine type (OH in 4, H in 1) are linked together in 1,1′-position by mild oxidation. Here is formed always a pair of isomerides, one of which belongs to the normal series and the other to the pseudo series. The series relation was determined. The authors assume the cause of the existence of the pair in the hindrance of free rotation of the linked molecules by the ethanamine chain. By Hofmann decomposition to the thebenone stage, both the normal and pseudo derivatives gave the same des-N-methyl-bases and thebenones. The ultra-violet absorption curves also showed the difference of normal and pseudo isomerides, but the identity of the des-N-methyl-bases and the thebenones from the both sources.

In linking in 1,1′-position of alkaloids of sinomenine type, we sometimes isolated a pair of isomerides and sometimes not. To the former group belonged sinomenine,[2] dihydrosinomenine[3] and *meta*-thebainone[4] and to the latter (+)-dihydrothebainone[5] and (+)-tetrahydrodesoxycodeine.[6] The latter two might have also formed a pair of bimolecular alkaloids, but one of the isomerides might have been amorphous and escaped our attention.

In carefully recrystallizing methiodide of bis-1,1′-(+)-tetrahydrodesoxycodeine (II), we noticed that there are two kinds of crystalls, one being short prisms and the other long. As these two are not interchangeable by repeated recrystallization from boiling water, it is clear that these two are quite different substances.

The criterium was given by the Clemmensen reduction of each component of two bis-1,1′-dihydrosinomenines (III). One component, which we call normal by reason given later, gave bis-1,1′-(+)-tetrahydrodesoxycodeine, whose methiodide crystallized in long prisms and the other, which we call ψ, gave bis-1,1′-(+)-tetrahydrodesoxycodeine whose methiodide crystallized in short prisms. Thus it is clear that bis-1,1′-(+)-tetrahydrodesoxycodeine, which we regarded formerly as a pure compound, was a mixture of normal and ψ-compounds. The ratio of the formation of these two in linking is nearly equal, as calculated from the quantity of each methiodide.

The same method may be applied to bis-1,1′-(+)-dihydrothebainone (IV). Up to present we obtained only one bis-1,1′-compound from (+)-dihydrothebainone. This bis-1,1′-compound formed well characterized hydrochloride and can be obtained in a pure state.

---

[1] 67th. Comm. on Sinomenine. The principal part of the theoretical was published in the *Proc. of Japan Academy*, Vol. **39**, No. 9 (1954).

[2] K. Goto, H. Sudzuki: *Bull. Chem. Soc. Japan*, **4**, 107 (1929).

[3] K. Goto: *ibid*., **4**. 129 (1929).

[4] K. Goto, Z. Kitasato: *Ann*. **481**. 81 (1930).

[5] K. Goto, H. Michinaka, H. Shishido: *Ann*. **515**, 297 (1935).

[6] K. Goto, H. Shishido: *Bull. Chem. Soc. Japan*. **10**, 262 (1935).

By reducing it by Clemmensen's method, we obtained a crystalline base, which formed exclusively methiodide of long prisms. On the other hand, the filtrate from the above hydrochloride, gave by the same reduction and treatment, a mixture of methiodides of short and long prisms. Thus the filtrate contained ψ-bis-1,1'-(+)-dihydrothebainone, whose hydrochloride is not easily crystallizable.

By this method we could determine clearly the series, to which these bimolecular alkaloids belong, as shown in the following list.

Table I

| | Linking of | Bis-1,1'-derivatives | | |
|---|---|---|---|---|
| 1) | Sinomenine | normal<br>↓ | | pseudo<br>↓ |
| 2) | Dihydrosinomenine | ←normal —<br>↓ | Na-Hg | pseudo ——→<br>↓ |
| 3) | Dihydrosinomeninol | normal | | pseudo |
| 4) | (+)-Dihydrothebainone | ←normal ←<br>↓ | | ψ(amorphous)→ |
| 5) | (+)-Dihydrothebainol | normal | Base mixture | ···· |
| 6) | (+)-Tetrahydrodesoxycodeine | →*n*-$ICH_3$ | | ψ-$ICH_3$ ←—— |

Note 1) Short, vertical arrows show catalytic reduction.
2) Long arrows on both sides indicate Clemmensen reduction.
3) Normal series forms crystallizable hydrochloride, but pseudo series does not.
4) Bis-1,1'-sinomenine, whose hydrochloride is crystalline, is natural and we call this series normal.

As to the cause of the existence of a pair of isomerides, we are now inclined to regard it as a similar, but a particular case to the stereoisomerism of substituted diphenyls. In these bis-1,1'-alkaloids, the model can easily show, the N-containing side chain hinders the free rotation of each half and we have the following two cases at the linking. In words, two molecules are linked together *en face* in one case, and *en dos* in the other case.

A——····B  A····——B
*I'*  *II'*

A and B are similarly constituted, but are not symmetrical in themselves. Therefore *I'* and *II'* can not be in the relation of subject and image. It is self-evident that each image of *I* and *II* must be prepared from morphine group, which is optical antipode of sinomenine. But when the formula *I* wants to become *II*, one of the component must rotate ca. 360 degree (in extreme case) and we imagine that this free rotation is greatly hindered by some reason hitherto unknown.

If this assumption is correct, then the distinction on *n*-and ψ-series must disappear when these alkaloids are converted into des-N-methyl-bases. This seems to be the case, at least so far we have examined, with *n*- and ψ-bis-1,1'-(+)-tetrahydrodesoxycodeines, and with *n*- and ψ-bis-1,1'-dihydrosinomenines.

The both bis-1,1'-derivatives gave the same bis-1,1'-des-N-methyl-base and the latter again the same tetrahydro derivatives. Bis-1,1'-dehydro-thebenane(−) and bis-1,1'-thebenane (+) from the both sources have the same m. p., the same specific rotatory power and show the same ultra-violet absorption curves.

A curious behavior of methiodides of *n*-and ψ-bis-1,1′-tetrahydrodesoxycodeine is that the both methiodides melt at the same degree and the mixed m.p. is not depressed. They show nearly the same specific rotation. This is true of the both methiodides of *n*- and ψ-bis-1,1′-dihydrosinomenine (Fig. 2 and 3). From these facts, one may suspect that these methiodides represent only the cases of polymorphism of crystalls. We maintain, however, that they are configuratively quite different from the following facts.

1. Original bases are different.
2. *n*- and ψ-methiodides are not interchangeable on repeated recrystallization from boiling water.
3. The difference in ultra-violet absorption.

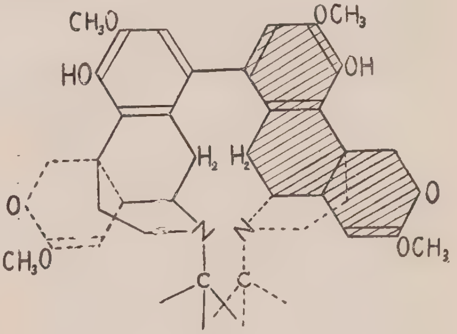


Fig. 1

I. n- and ψ-Disinomenine (-CO-C($OCH_3$)=)
II. Bis-1,1′-(+)-tetrahydrodesoxycodeine (-$CH_2$-$CH_2$-)
III. Bis-1,1′-dihydrosinomenine (CO-CH($OCH_3$)-)
IV. Bis-1,1′-(+)-dihydrothebainone (-CO-$CH_2$-)

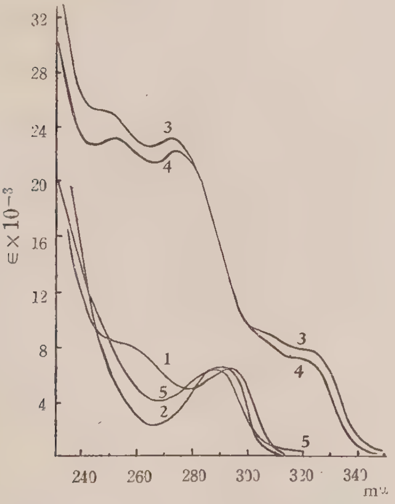


Fig. 2. Bis-1,1′-(+)-tetrahydrodesoxycodeine
1. *n*-methiodide
2. ψ-methiodide
3. Bis-1,1′-des-N-methyl-base (*n*- and ψ-)
4. Bis-1,1′-D-dehydro-thebenane (*n*- and ψ-)
5. Bis-1,1′-D-thebenane (*n*- and ψ-)

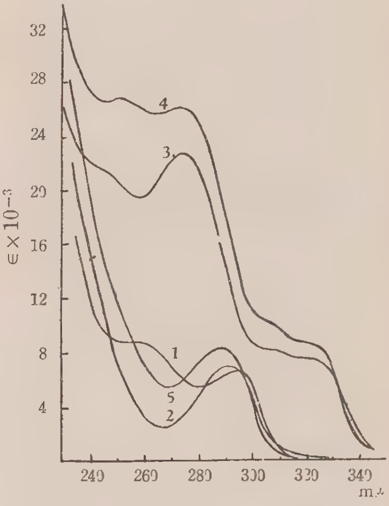
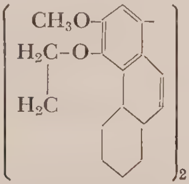


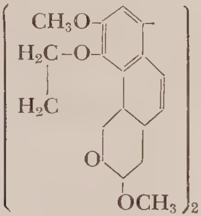
Fig. 3. Bis-1,1′-dihydrosinomenine
1. *n*-methiodide
2. ψ-methiodide
3. Bis-1,1′-des-N-methyl-base (*n*- and ψ-)
4. Bis-1,1′-7-methoxy-D-dehydro-thebenone (*n*- and ψ-)
5. Bis-1,1′-7-methoxy-D-thebenone(*n*- and ψ-)

The comparison of the ultra-violet absorption curves showed clearly the difference of both isomerides. *n*-Derivatives, as methiodides, are more hyperchromic and *ψ*-derivatives are more hypochromic. On the contrary, the same measurement revealed the coincidence of the curves, when these *n*- and *ψ*-methiodides are turned into their respective des-*N*-methyl-bases, dehydrothebenane and thebenane. Our hypothesis on the origin of these two isomerides in bis-1,1′-derivatives seems therefore to have been verified to some degree by these spectroscopic observation.

Our hearty thanks are due to Sankyo Pharmaceutical Co. for microanalyses and to Ministry of Education for a grant to this investigation.



VII Bis-1,1′-dehydro-thebenane
VIII Bis-1,1′-thebenane ($\Delta^{9:10}$ in VII saturated)



XII Bis-1,1′-7-methoxy-dehydro-thebenone
XIV Bis-1,1′-7-methoxy-thebenone($\Delta^{9:10}$ in XII is saturated).

## *Experimental*

## Part I

### (1) Separation of *n*- and *ψ*- methiodide of bis-1,1′-(+)-tetrahydrodesoxycodeine (II)

The mixture of *n*- and *ψ*- bis-1,1′-(+)-tetrahydrodesoxycodeine, as extracted by chloroform, was dissolved in its weight of methanol and added with enough methyliodide. The liquid warmed itself and deposited methiodides quickly. They are filtered and recrystallized from twenty times its weight of boiling water. *ψ*-Methiodide crystallized out in heavy, short prisms first and *n*-methiodide in light, long prisms later. They are separated by mechanically and by repeated recrystallization. M.p. of *n*- methiodide 272°. $[\alpha]_D^{16}=+73.7°$ (c 0.1803, $H_2O$). M.p. of *ψ*-methiodide 272° also. $[\alpha]_D^{16}=+64.7°$ (c 0.400, $H_2O$). Mixed m.p. is, however, unaltered. This curious behavior may suggest the transformation of one form to the other more stable form by heat before melting.

(*Anal.* Calcd. for $(C_{19}H_{27}O_2NICH_3)_2$: N, 3.27. Found: N, 3.13 (for normal); N, 3.08 (for pseudo).

### (2) Hofmann decomposition of the both methiodides; Bis-1,1′-des-N-methyl-tetrahydrodesoxycodeine (V, derived from II)

(A) n-*Methiodide.* The best condition of decomposing is to boil *n*-methiodide with ten times its weight of 25 per cent. aqueous potassium hydroxide gently for 1.5 hours. The layer of des-N-methyl-base, which becomes semi-solid, when cooled, is separated and dissolved in dil. hydrochloric acid. The free des-N-methyl-base is taken up in chloroform, from which it crystallizes out on evaporation of the solvent. It is white hairy microscopic crystalls, when washed thoroughly with methanol. M.p. 247° (sharp). $[\alpha]_D^{8}=+57.3°$ (c 0.6406, 1% HCl).

(*Anal.* Calcd. for $(C_{19}H_{26}O_2N)_2$: C, 75.96; H, 8.72; N, 4.66. Found: C, 71.26; H, 7.22; N, 4.18).

(B) *ψ-Methiodide.* The condition of the decomposition is same as above. Only ψ-methiodide is precipitated as Na-salt in the above concentration of KOH. The ψ-des-N-methyl-base melts at 247°. Also the mixed m.p. with the *n*-des-N-methyl-base (from A). $[\alpha]_D^{26} = +57.6°$ (c 0.2205, 1% HCl).

### (3) Tetrahydro-bis-1,1′-des-*N*-methyl-(+)-tetrahydrodesoxycodeine (VI, derived from V)

By catalytic reduction of the above bis-1,1′-des-N-methyl-base in glacial acetic acid, using $PtO_2$ as catalyst, gave

(A) *n*-Series. Tetrahydro derivative of m.p. 262°, $[\alpha]_D^{22} = +16.1°$ (c 0.112, 1% HCl)

(B) ψ-Series. Tetrahydro derivative of m.p. 261°, $[\alpha]_D^{21} = +16.0°$ (c 0.244, 1% HCl)

(C) The same tetrahydro-bis-1,1′-des-N-methyl-(+)-tetrahydrodesoxycodeine can be obtained by linking together of dihydro-des-N-methyl-(+)-tetrahydrodesoxycodeine. (On linking condition see further. The monomolecular starting material can also be obtained by Clemmensen reduction of des-N-methyl-dihydrosinomenine). M.p. 263°, $[\alpha]_D^{20} = +15.8°$ (c 0.494, 1 % HCl).

(*Anal.* Calcd. for $(C_{19}H_{28}O_2N)_2$: C, 75.45; H, 9.33; N, 4.63. Found: C, 75.17; H, 8.79; N, 4.46). Mixed M.p. A+B, 261°; A+C, 262°; B+C, 261°.

### (4) Bis-1,1′-D-dehydro-thebenane (VII)

(A) Methiodide of V (*n*-series) was decomposed by boiling with 25% KOH solution (10∼20 times) for 30 min. The separated solid was dissolved in little methanol and poured into a large quantity of ether. Ether was washed several times with dilute caustic alkali, dilute HCl and water until colourless. The residue of ether is dissolved in a small quantity of glacial acetic acid, from which bis-1,1′-dehydro-thebenane crystallized out in colourless, long prisms. M.p. 185° (bubble formation, but no meniscus was formed even at 225°). With conc. $H_2SO_4$, yellow green, but violet on the next day. $[\alpha]_D^{19} = -210°$ (c 0.280 chlf.).

(B) Methiodide of V (ψ-series) gave the same substance by the same treatment. M.p. 185°. $[\alpha]_D^{20} = -210.7°$ (c 0.262 chlf.). Same colour reaction as above.

(*Anal.* Calcd. for $(C_{17}H_{19}O_2)_2 + 2H_2O$: C, 74.69; H, 7.74. Found: C, 74.48; H, 6.76).

### (5) Bis-1,1′-D-thebenane (VIII)

This substance can be obtained whether by the Hofmann decomposition of VI or by catalytic reduction of VII.

(A) From *n*-series. M.p. 215°. $[\alpha]_D^{24} = +35.1°$ (c 0.268, chlf.).

(B) From ψ-series. M.p. 214°. $[\alpha]_D^{21} = +35.2°$ (c 0.125, chlf.).

(C) From linked substance. M.p. 215°. $[\alpha]_D^{23} = +34.6°$ (c. 0.480, chlf.).

In conc. $H_2SO_4$, the bis-1,1′-D-thebenane dissolves almost colourless at first, but the solution takes a beautiful blue colour on next day.

(*Anal.* Calcd. for $(C_{17}H_{21}O_2)_2$: C, 79.34; H, 8.22. Found: C, 79.14: H, 8.40).

## Part II

### (6) Clemmensen reduction of *n*- and ψ- bis-1,1′-(+)-dihydrothebainone (IV)

(A) From *n*-series. Hydrochloride of IV (1.2 g) was reduced with Clemmensen's Zn-Hg, 20% HCl (10cc) and conc. HCl (6 cc) for 2∼5 hours on a steam bath. The $ZnCl_2$-double salt appeared, while hot, and increased when added with water (60 cc). From this double salt, 0.3 g nice, white crystalls was obtained. M.p. 245° (with foaming). $[\alpha]_D^{24} = +159.0°$ (c 0.967, chlf.). It gave only methiodide of long prisms (m.p. 272°).

(B) From *ψ*-series. The same reduction of the filtrate from the hydrochloride of *n*- IV gave amorphous base, which was dissolved in 5 cc methanol and precipitated partly by adding 20cc ether. The base, remained in ether, was not crystallizable, but gave only methiodide of short prisms. The precipitated part gave chiefly methiodide of long prisms.

### (7) Clemmensen reduction of bis-1,1′-dihydrosinomenine (III).

(A) n-*Derivative.* From 3g of the hydrochloride of III, 0.6g crystalline base was obtained. M.p. 245°. $[\alpha]_D^{24} = +158.6°$ (c 0.420, chlf.). It gave only methiodide of long prisms.

(B) *ψ-Derivative.* From 1g of the free base, 0.1g crystalline base was obtained. M.p. 242° from methanol. It was changed into only methiodide of short prisms.

### (8) *n*- and *ψ*- Bis-1,1′-dihydrosinomeninol (IX)

By linking dihydrosinomeninol (m.p. 167°) with $AgNO_3$, we obtained also *n*- and *ψ*-substance. The series relation was easily determined by catalytic reduction of *n*- and *ψ*-bis-1,1′-dihydrosinomenine (III) respectively.

(A) Dihydrosinomeninol (2g, 1mol.) was dissolved in methanol (20cc), and poured into water (100 cc). To this fine suspension, 100 cc of aqueous solution of $AgNO_3$ (1.06 g, 1mol.) were added. After standing 80 min, silberion was precipitated by table salt and the whole was filtered. Isolation as usual. The residue, obtained by evaporation of chloroform, was recrystallized from methanol. First crop consisted of long prisms and amounted to 1.2 g. M.p. 215°. $[\alpha]_D^{22} = +52.96°$ (c 1.552, chlf.). The mother liquor gave short prisms, which melted at 290° (dec.). Yield 0.3 g. $[\alpha]_D^{22} = +83.19°$ (c 0.952, chlf.).

(B) Catalytic reduction of *n*-bis-1,1′-dihydrosinomenine (III) gave only bis-1,1′-dihydrosinomeninol of m.p. 215° (Also the mixed m.p. with the corresponding substance from A). Whereas catalytic reduction of *ψ*-bis-1,1′-dihydrosinomenine gave short prisms of m.p. 290° (Also the mixed m.p.).

(*Anal.* Calcd. for $(C_{19}H_{26}O_4N)_2$ (664): N, 4.22. Found: for *n*-der. N, 4.21, for *ψ*-der. N, 4.13). Methiodide of *n*-derivative. M.p. 268° (dec.).

### (9) Bis-1,1′-(+)-dihydrothebainol (X)

(A) 2.3g (+)-dihydrothebainol (m.p. 165°. Catalytic preparation) were linked together by $AgNO_3$. Raw yield 1.8g. But, by recrystallization from methanol diminished the yield to 0.2 g. (This suggests perhaps the formation of another isomeride.). Pale yellow prisms. M.p. 246°~248°. $[\alpha]_D^{25} = +86.8°$ (c 0.4908, $C_2H_5OH$).

(B) *n*-Bis-1,1′-(+)-dihydrothebainone (1.5g) was dissolved in methanol (50cc) and reduced catalytically, using $PtO_2$ (0.1 g) as catalyst. Absorption of $H_2$ was complete in 25 min. Beautiful prisms from methanol. M.p. 248°~250° (sintering at 223°). The mixed m.p. of (A) and (B) was also 248°. $[\alpha]_D^{25} = +88.2°$ (c 0.9256, $C_2H_5OH$).

(*Anal.* Calcd. for $(C_{18}H_{24}O_3N)_2$: N, 4.68. Found: N, 4.77).

Thus it is clear that the bis-1,1′-(+)-dihydrothebainol of m.p. 248° belongs to *n*-series.

*Methiodide.* M.p. 269°. $[\alpha]_D^{25}=+67°$ (c 0.1352, $H_2O$).

(*Anal.* Calcd. for $(C_{18}H_{24}O_3N \cdot ICH_3)_2$: I, 28.60. Found: I, 28.91).

*Tetracetyl-derivative.* *n*-Bis-1,1′-(+)-dihydrothebainol (0.4g), sodium acetate (0.5g) and acetic anhydride (5cc) were heated on steam-bath for 3 hours. Isolation as usual. Prisms from ether. Yield good. M.p. 237° (sintering at 233°). $[\alpha]_D^{25}=+66°$ (c 0.696, chlf.).

*Methiodide of tetracetyl derivative.* Tetracetyl derivative was dissolved in methanol and added with methyl iodide. On adding ether to the mixture, the methiodide crystallized out slowly in bundles. M.p. 275° (dec.). Very soluble in water and methanol.

(*Anal.* Calcd. for $(C_{22}H_{28}O_5 \cdot ICH_3)_2$: $4CH_3CO$ 16.3. Found: 17.3).

## Part III

### (10) *n*- and *ψ*- Bis-1,1′-dihydrosinomenine (III)

As reported already, linking of dihydrosinomenine with silver nitrate gives normal and pseudo bis-1,1′-derivatives. The normal derivative is identical with that obtained by the catalytic reduction of normal (natural) disinomenine. The property of *n*- and *ψ*-bis-1,1′-dihydrosinomenines is given in the following lines[7].

Normal. Rhombic plates or prisms. Ca. 2.5% soluble in alc., >10% soluble in chloroform. M.p. 247°[2]. $[\alpha]_D^{7}=+264.41°$. HCl-salt crystalline.

Pseudo. Hairy needles. Sol. in alc. ca. 2.5%. Sol. in chloroform <2%. M.p. 271°. $[\alpha]_D=+167°$. HCl-salt amorphous.

### Property of the methiodides.

Normal. From water, hairy needles at first, but after several hours, they begin to change form, stout, short prisms on the next day. M.p. 273° (sharp). $[\alpha]_D^{17}=+84.2°$ (c 0.5332, $H_2O$).

Pseudo. Slender long prisms. No change of crystall form. M.p. 273° (sharp). Also the mixed m.p. with the above. $[\alpha]_D^{16}=+84.6°$ (c 0.8293, $H_2O$).

From M.p. and specific rotation, the both methiodides might seem at first sight to be identical, but we think they are quite different, judged from their behaviour on recrystallization and from the difference in original bases (see, also the Fig. 3).

### (11) Bis-1,1′-des-N-methyl-dihydrosinomenine (XI)

We obtained the same des-N-methyl-base from normal and pseudo substances, thus,

*n*-des-N-Methyl-base. M.p. 239°. $[\alpha]_D^{7}=+52.1°$ (c 0.259, 1% HCl).

*ψ*-des-N-Methyl-base. M.p. 238°. $[\alpha]_D^{7}=+54.8°$ (c 0.4538, 1% HCl). Mixed m.p. 238°.

The colour reaction against conc. $SO_4H_2$ is green at first, and violet on the next day.

The Hofmann decomposition was carried out in the following way.

*n*-Methiodide (4g) was boiled with 25% KOH (50g) for one hour. The des-N-methyl-base solidified while hot, and was filtered through a glass filter. It crystallized out from chloroform solution, when evaporated. The hairy crystalls were washed thoroughly with methanol. Colourless hairs, but slowly take a brown colour in the air.

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[7] Some of these properties were already given in, K. Goto and H. Sudzuki: *Bull. Chem. Soc. Japan*, **4**, 107 (1929).

[8] Observed several weeks after the recrystallization. Directly after, it gives oftec much higher m.p.

(*Anal.* Calcd. for $(C_{20}H_{26}O_4N)_2$: C, 69.74; H, 7.61; N, 4.07. Found: C, 68.87; H, 7.13; N, 4.20).

The ψ-methiodide was also decomposed in the same way.

### (12) Bis-1,1′-7-methoxy-D-dehydrothebenone (XII)

The same substance was obtained from *n*- and ψ-des-N-methyl-bases.

Normal-D-thebenone. M.p. 225° (sintering at 195°). $[\alpha]_D^{12}=-265.8°$ (c 1.122, chlf.).

Pseudo-D-thebenone. M.p. 227° (sintering at 198°). $[\alpha]_D^{16}=-265.5°$ (c 0.380, chlf.)

Preparation. The amorphous methiodide of *n*-des-N-methyl-base (4 g) was boiled with 25 % KOH (20 g) for 30 min. The crust formed on the solution was dissolved in methanol (10cc) and poured into ether (200 cc). The ethereal solution was decolourized completely, when washed with caustic alkali, dilute hydrochloric acid, and water. The residue of ether evaporation crystallized out spontaneously. It can be purified by dissolving in a small quantity of glacial acetic acid and adding methanol to it. Long prisms. Yield ca. 0.5 g (15 %).

(*Anal.* Calcd. for $(C_{18}H_{19}O_4)_2$: C 72.22; H, 6.40; $OCH_3$, (two) 20.74. Found: C, 71.97; H, 6.47: $OCH_3$, 20.49).

### (13) Bis-1,1′-dihydro-des-N-methyl-dihydrosinomenine (XIII)

As it was very difficult to reduce des-N-methyl-base of bis-1,1′-dihydrosinomenine catalytically in ordinary condition, this dihydro-base was prepared by the linking of dihydro-des-N-methyl-dihydrosinomenine by silver nitrate. The obtained bis-1,1′-dihydro-des-N-methyl-base can be recrystallized from methanol. M.p. 237°. $[\alpha]_D^{15}=+7.26°$ (c 1.047, chlf.).

(*Anal.* Calcd. for $(C_{20}H_{28}O_4N)_2$: C, 69.34; H, 8.15; N, 4.04. Found: C, 68.96; H, 7.99; N, 4.28).

### (14) (−)-Bis-1,1′-7-methoxy-D-thebenone (XIV)

(A) From the methiodide of the above bis-1,1′-dihydro-des-N-methyl-base. The experimental condition was the same as described with I-dehydro-thebenone. Slender prisms from methanol. M.p. 245°. Yield good. Halochromy in conc. $H_2SO_4$: pale yellow, green and deep blue on the next day. $[\alpha]_D^{9}=-52.6°$ (c 1.620, chlf.).

(B) By the catalytic reduction of the dehydro-D-thebenone (100 mg) in glacial acetic acid (15cc) with $PdCl_2$ (60 mg) and hydrogene for 30 min., could be obtained the same bis-1,1′-7-methoxy-D-thebenone as in (A). M.p. 224°. Mixed m.p. with (A) also 224°.

(C) Catalytic reduction of the D-dehydrothebenone from ψ-substance gave also l-thebenone of m.p. 245°. Mixed m.p. with (A) 245°. $[\alpha]_D^{17}=-51.2°$ (c 0.234, chlf.).

(*Anal.* Calcd. for $(C_{18}H_{21}O_4)_2$: C, 71.74; H, 7.02. Found: C, 71.17, H, 6.82).

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# 2. *Studies on Lactic Acid Fermentation.*

## *Part 1. Metabolism of Pyruvic Acid by Hetero-type Lactic Acid Bacteria.*

By Hideo KATAGIRI and Kazutami IMAI

The metabolism of pyruvate by hetero-fermenters was found to vary according to experimental conditions; acetic acid formation was increased under aerobic conditions, while anaerobically acetoin formation was accelerated.

When pyruvate was added to the growing culture during the early stage of growth, it was reduced to lactic acid, while acetoin was obtained from pyruvate when it was added during the later stage of the growing culture.

No relationship of acetaldehyde to acetoin formation was ascertained, and no evidence was obtained on ethanol formation from pyruvate.

### *Introduction*

In nature, formation of lactic acid is commonly seen, namely in muscle or in various microorganisms. As for lactic acid bacteria, they are divided into two groups; one is a homotype which produces lactic acid as the sole product of sugar fermentation, and the other is a hetero-type which produces carbon dioxide and ethanol or acetic acid in addition to lactic acid. The mechanism of lactic acid fermentation by bacteria has not yet been clarified. It has been shown by De Moss et al.,[1] that the fermentation of glucose by a hetero-type lactic acid bacteria would not proceed via the Embden-Meyerhof scheme, but no other scheme of the mechanism has yet been put forward.

In order to get some evidence on chemical changes involved in the fermentation, we have studied the metabolism of pyruvate by hetero-type lactic acid bacteria.

### *Experimental and Results*

The microorganism used in this report is isolated from cheese by us, and identified as *Lactobacillus fermentum* $\beta$ (hetero-type).

Pyruvic and lactic acid were determined by the methods of Friedemann and Haugen[2] and of Friedemann et al.,[3] respectively. Acetoin was determined by the method of Van Niel.[4] The bacterial cells grown in malt extract (containing 5% $CaCO_3$) for 48 hours were harvested by Sharpless ultracentrifuge. These intact cells were used for experiments.

Pyruvic acid is considered the mother

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[1] D. De Moss, R. C. Bard and I. C. Gunsalus, *J Bact.*, **62**, 499 (1951).

[2] T.E. Friedemann and G. E. Haugen, *J. Biol. Chem.*, **147**, 415 (1943).

[3] T.E. Friedemann, M. Cotonio and P.A. Shaffer, *J. Biol. Chem.*, **73**, 335 (1927).

[4] Van Niel, *Biochem. Z.*, **187**, 472 (1927)

substance of lactic acid, but controversial discussions are put forward as to whether ethanol is derived from pyruvic acid even in the case of hetero-type lactic acid bacteria as was already ascertained by yeast. Several reaction formulas have hitherto been proposed for bacterial metabolism of pyruvate. For instance:

(1) $CH_3 \cdot CO \cdot COOH \longrightarrow CH_3 \cdot CHO + CO_2$ Carboxylase

(2) $CH_3 \cdot CO \cdot COO^- + 1/2\,O_2 \longrightarrow CH \cdot COO^- + CO_2$ *Pr. vulgaris*[5]

(3) $CH_3 \cdot CO \cdot COO^- + (OH)_2 \cdot PO_2^- + O_2 \rightarrow CH_3 \cdot COOPO_3^{--} + CO_2$ *L. delbruckii*[6]

(4) $CH_3 \cdot CO \cdot COO^- + (OH)_2 \cdot PO_2^- \rightarrow CH_3 \cdot COOPO_3^{--} + HCOO^- + H^+$ *E. coli*[7]

(5) $CH_3 \cdot CO \cdot COO^- + (OH)_2 \cdot PO_2^- \rightarrow CH_3 \cdot COOPO_3^{--} + H_2 + CO_2$ *Cl. butylicum*[8]

(6) $CH_3 \cdot CO \cdot COOH + DPN + CoA \longrightarrow \text{Acetylphosphate} + CO_2 + DPNH_2$ *E. coli*[9]

(7) $2\,CH_3 \cdot CO \cdot COOH + H_2O \longrightarrow CH_3 \cdot CHOH \cdot COOH + CH_3 \cdot COOH + CO_2$ *Gonococcus* and *Staphylococcus*[10]

(8) $CH_3 \cdot CO \cdot COOH + H_2 \longrightarrow CH_3 \cdot CHOH \cdot COOH$ Lactic dehydrogenase

(9) $2\,CH_3 \cdot CO \cdot COOH \longrightarrow 2\,CO_2 + CH_3 \cdot CHOH \cdot CO \cdot CH_3$ *A. aerogenes*[11]

The formula (1) is a well known reaction occuring in yeast, but no convincing demonstration of this reaction was obtained by ethanol forming bacteria, except *Termobacterium mobile* and *Sarcina ventriculi*,[12] since presence of carboxolase in lactic acid bacteria was never verified.

Formulas (2) to (7) are the reactions producing acetic acid, and are divided into two types: one needs phosphate and the other proceeds without phosphate. Formula (7) represents the reaction in which hydrogen generated from pyruvate is transported to the other pyruvate molecule and thence lactic acid is formed. It is noteworthy that in formula (9) of the acetoin-forming system shown by Silverman and Werkman,[11] carboxylase is not implicated. It is indisputable to point out that there are some reaction systems other than these listed above, for example, Wood-Werkman's reaction or malic enzyme system of fixing carbon dioxide to pyruvate.

## 1. Manometric experiments

Using the Warburg manometer, we observed the pyruvate metabolism of cell suspensions. The composition of the reaction mixture is as follows: Na-

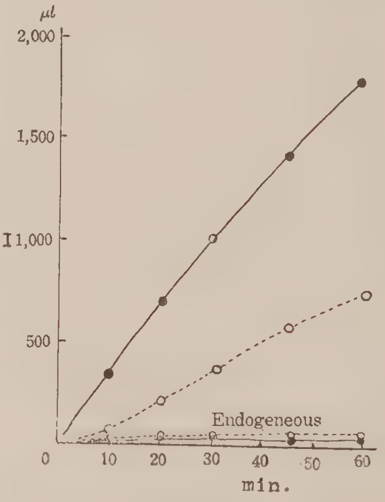

Fig. 1. Gaseous Metabolism of Pyruvate by cell Suspensions

•—• Carbon dioxide evolution

○····○ Oxygen uptake

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[5] P.K. Stumpf, *J. Biol. Chem.*, **159**, 529 (1945).

[6] F. Lipmann, *Nature*, **144**, 381 (1939).

[7] G. Kalnitsky and C.H. Werkman, *Arch. Biochem.*, **2**, 113 (1943).

[8] H. J. Koepsell and M. J. Johnson, *J. Biol. Chem.*, **145** 379 (1951).

[9] S. Korkes, A. del Campillo, I.C. Gunsalus and S. Ochoa, *J. Biol. Chem.*, **193**, 721 (1951).

[10] H.A. Krebs, *Biochem J.*, **31**, 661 (1937).

[11] M. Silverman and C.H. Werkman, *J. Biol. Chem.*, **138**, 35 (1941).

[12] J.B. Sumner and K. Myrbäck, *The Enzymes*, Vol. II, 824 (1952).

pyruvate 0.08 M, $Mn^{++}$ 27.5 $\gamma$, $Mg^{++}$ 610 $\gamma$ (as chloride), cocarboxylase 40$\gamma$, M/15 phosphate buffer (pH 6.4) of 0.8 ml and cell suspension (intact cells of 20 mg dry weight); total volume is 2.5 ml, the temperature kept at 37°C and the gas phase is air. The results are shown in Fig. 1 in which we observed oxygen uptake and carbon dioxide evolution. In the reaction mixture we were able to determine acetoin as Ni-dimethylglyoxime, and to detect lactic[13] and acetic acids[14] by paper partitionchromatography. From these results, decomposition of pyruvate by a cell suspension was clearly demonstrated, however, at least three reactions such as pyruvic oxidation system, the acetoin forming system and the lactic dehydrogenase system would be supposed to be related to the decomposition of pyruvate. When phosphate buffer is substituted by citrate, carbon dioxide evolution and oxygen uptake are greately diminished as shown in Fig. 2, so that phosphate would play an important role in the pyruvic oxidation system.

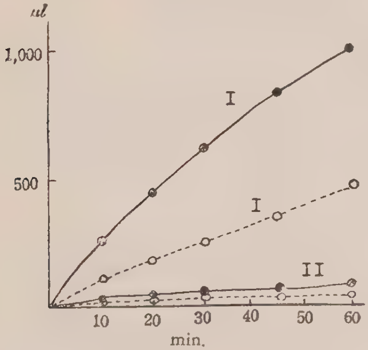

Fig. 2. Necessity of Phosphate

•——• Carbon dioxide evolution
o····o Oxgen uptake
I····· Phosphate buffer
II······Citrate buffer

Necessity of phosphate in the pyruvic oxidation system is again ascertained by the results shown in Fig. 3, in which citrate has no inhibitory effect, but reveals slight activation of this system. It is worthwhile to point out that no noticeable effect on this system was ever detected with Ca-pantothenate of 50 $\gamma$/2.5 ml nor 2,4-dinitrophenol of 1/1000 M.

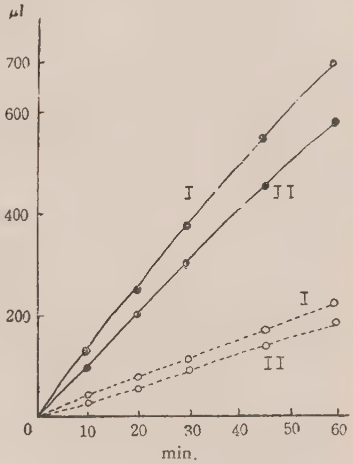

Fig. 3. Effect of Citrate

•——• Carbon dioxide evolution
o····o Oxygen uptake
I·······Phosphate plus citrate buffer
II······Phosphate buffer

## 2. Macro experiments

As was shown in the preceding experiments we deduced the existence of three systems, in the pyruvate metabolism; macro experiments are carried out in order to get a clear idea of the relationship among these systems.

The scale of the reaction mixture is about twenty times as great as that of the manometric experiments and it contained Na-pyruvate 400mg, cocarboxylase 800 $\gamma$, $Mg^{++}$ 12 mg, $Mn^{++}$ 0.55mg, 15 ml of M/15 phosphate buffer (pH 6.4) and the cell suspension (intact cells of 355 mg dry weight) in a total volume

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13) J.W.H. Lugg and B.T. Overell, *Nature*, **160**, 87 (1947).

14) E.P. Kennedy and H.A. Barker, *Anal. Chem.*, **23**, 1033 (1951).

of 50 ml. Incubation period was three hours at 37°C.

A) Reaction under aerobic and anaerobic conditions.

The reactions were proceeded as follows:

No. 1—anaerobic (filled with $H_2$ gas and incubated statically).

No. 2—aerobic (kept in the air and agitated).

At the end of reaction the mixture was analyzed, and pyruvic, acetic, and lactic acids, and acetoin were determined. The results are shown in Table I in which it is clearly pointed out, that under aerobic conditions (No. 2), acetic acid formation is increased, while formation of lactic acid and acetoin is decreased.

Table I

Metabolism of Pyruvate under Aerobic and Anaerobic Conditions

| | Anaerobic | Aerobic |
|---|---|---|
| Pyruvate consumed (%) | 65.9 | 61.6 |
| Acetoin formed (%) | 22.5 | 15.4 |
| Acetic acid formed (%) | 6.2 | 21.8 |
| Lactic acid formed (%) | 22.3 | 10.1 |

B) Effect of riboflavin

Under aerobic conditions we tested the effect of riboflavin and of methylene blue. In this case anaerabic conditions are brought about by evacuation.

As is shown in Table II, methylene blue (1/10,000 M) reveals almost no effect, but the addition of riboflavin (1/10,000M) results in an increasing effect on acetic and lactic acid formation and a noticeable decrease of acetoin formation.

Table II

Effect of Riboflavin

| | Cont. | Mb | $B_2$ |
|---|---|---|---|
| Pyruvate consumed (%) | 57.8 | 54.7 | 56.3 |
| Acetoin formed (%) | 23.4 | 26.6 | 19.3 |
| Acetic acid formed (%) | 12.1 | 12.8 | 20.0 |
| Lactic acid formed (%) | 12.4 | 18.1 | 18.7 |

C) Acetoin metabolism

As was shown in the preceding experiment, this microorganism forms a large quantity of acetoin. It is important to investigate whether acetoin is a normal intermediate product of heterotype lactic acid fermentation.

Tomiyasu[15] reported that certain bacteria could split acetoin into acetic acid and ethanol, so that the reaction mixture was kept for as long as 48 hours in order to find out whether the formed acetoin would be decomposed in the course of time. The results are shown in Table III in which there is no evidence of degradation of acetoin since the yield of acetoin was found to be constant (29.2 and 25.3 %) despite incubation periods (3 and 48hrs.

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[15] Y. Tomiyasu, *J. Agr. Chem. Soc. Japan*, **14**, 634 (1938).

Table III

Consumption of Acetoin

| Incubation period (hr.) | 3 | 48 |
|---|---|---|
| Pyruvate consumed (%) | 51.2 | 94.6 |
| Acetoin formed (%) | 29.2 | 25.3 |

respectively). The mechanism of bacterial formation of acetoin is not yet clear. Silverman and Werkman[11] reported that acetaldehyde is not an intermediate product in the reaction of Aerobacter, while Happold and Spencer[16] deduced a reaction in which acetaldehyde-enzyme complex and acetate will condence to diacetyl and then will be reduced to acetoin. No relationship of acetaldehyde to acetoin formation was ascertained by the results shown in Table IV, in which acetaldehyde or dimedon was added to the reaction mixture containing Na-pyruvate 240mg, cocarboxylase 800 $\gamma$, $Mg^{++}$ 12 mg, $Mn^{++}$ 0.55 mg, 15 ml of M/15 phosphate buffer (pH 6.4) and the cell suspension (intact cells of 355 mg dry weight) in a total volume of 15 ml. The mixture was placed in a 55 ml Erlenmeyer flask and incubated statically (not strictly anaerobic conditions), acetaldehyde or dimedon was added, 130 or 100 mg/50 ml respectively.

Table IV

Effect of Dimedon and Acetaldehyde

| | Dimedon | Acetald. | Cont. |
|---|---|---|---|
| Pyruvate consumed (%) | 73.3 | 69.6 | 60.8 |
| Acetoin formed (%) | 27.3 | 26.5 | 26.4 |
| Acetic acid formed (%) | 20.0 | 30.0 | 21.0 |
| Lactic acid formed (%) | 12.0 | 16.1 | 13.7 |

### 3. Experiments on growing culture

To investigate the effect of acetaldehyde or alcohol on a normal growing culture, one of them was added to the cultures (malt extract, containing 5 per cent $CaCO_3$) of 24 hours' incubation.

Table V

Effect of Acetaldehyde, Ethanol, and Dimedon on a Growing Culture

| | Acetald. | Ethanol | Dimedon | Cont. |
|---|---|---|---|---|
| Initial concentration of sugar (as maltose) (g) | 3.90 | 3.90 | 3.90 | 3.90 |
| Concentration of additions (g/100 ml) | 1.3 | 1.88 | 0.3 | — |
| Sugar consumed (%) | 75.6 | 85.4 | 88.7 | 86.6 |
| Lactic acid formed (% on consumed sugar) | 51.8 | 48.6 | 44.7 | 51.9 |
| Ethanol formed (%) | 36.1 | 21.3 | 20.8 | 25.1 |
| Acetic acid formed (%) | 9.7 | 2.5 | 1.7 | 2.6 |
| Formation of acetoin | — | — | — | — |

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[16] F.C. Happold and C.P. Spencer, *Biochim. et Biophys. Acta*, **8**, 543 (1952).

At the same time we inoculated the seed of bacteria to the malt extract containing dimedon and incubated at 37°C for 72 hours in each case. At the end of fermentation, cultures were analyzed, and the results are shown in Table V. From the Table it is observed that alcohol has no effect on the ratio of fermentation products, while acetaldehyde reveals an increase on the formation of alcohol and acetate.

However, it is difficult to conclude that acetaldehyde is a precursor of ethanol or acetate, because dimedon has no effect on the fermentation of sugar, and furthermore the subsequent, experiment does not show any evidence for formation of alcohol from pyruvate.

In order to investigate pyruvate metabolism in a growing culture, experiments in which Na-pyruvate (320 mg per 50 ml) was added to the culture medium (malt extract containing 4 % $CaCO_3$) after requisite period of incubation, and incubated again for an additional three hours at 37°C and then analyzed, were carried out. These results are shown in Table VI in which it will be seen that almost all the pyruvate added was consumed even in three hours and was converted solely to lactate when pyruvate is added to a young culture of 24 hours' incubation. The pyruvate which was added to the culture of fourtyeight hours' incubation was converted mainly to lactate and then to acetoin, about 45 per cent of the consumed pyruvate is considered to be converted to lactate and about 35 per cent is suggested to be used for the formation of acetoin (assuming that 1mol. of acetoin is formed from 2mol. of pyruvate;

$$2\,CH_3 \cdot CO \cdot COOH \longrightarrow CH_3 \cdot CHOH \cdot CO \cdot CH_3 + 2CO_2).$$

On the other hand, consumption of

Table VI

Pyruvate Metabolism in Growing Culture

| Incubation period (hr.) | 20 | | 48 | | 72 | |
|---|---|---|---|---|---|---|
| Initial concentration of sugar (g/50 ml; as maltose) | 1.80 | 1.80 | 1.80 | 1.80 | 1.80 | 1.80 |
| Consumed sugar (g/50 ml) | 1.15 | 1.10 | 1.50 | 1.45 | 1.70 | 1.70 |
| Sugar consumed (%) | 63.8 | 61.1 | 83.3 | 80.6 | 94.4 | 94.4 |
| Addition of pyruvic acid (mg/50 ml) | — | 320 | — | 320 | — | 320 |
| Consumed pyruvic acid (mg/50 ml) | — | 320 | — | 310 | — | 182 |
| Consumption of added pyruvic acid (%) | — | 100 | — | 96.9 | — | 56.9 |
| Lactic acid formed (mg/50 ml) | 505 | 830 | 866 | 983 | 858 | 858 |
| Formation of lactic acid (% on consumed sugar) | 43.9 | (75.5) | 57.7 | (67.8) | 50.5 | (50.5) |
| Ethanol formed (mg/50 ml) | 275 | 270 | 379 | 368 | 414 | 403 |
| Formation of ethanol (% on consumed sugar) | 23.9 | 24.5 | 25.3 | 24.5 | 24.4 | 2.37 |
| Acetic acid formed (mg/50 ml) | 30 | 57 | 30 | 66 | 40 | 50 |
| Formation of acetic acid (% on consumed sugar) | 2.6 | (5.2) | 2.0 | (4.6) | 2.4 | (2.9) |
| Acetoin formed (mg/50 ml) | 0 | 10 | 0 | 54 | 0 | 53 |
| Formation of acetoin (% on consumed pyruvic acid) | 0 | 3.1 | 0 | 17.4 | 0 | 29.1 |

pyruvate added to seventy-two hours, culture is found to be only 57 per cent, and the majority of the consumed pyruvate is used for the formation of acetoin (about 60 per cent). It is important to point out that added pyruvate is never converted to alcohol, since formation of alcohol is constant through out the experiments. From these results we deduced that pyruvate cannot act as a precursor of alcohol in hetero type lactic acid fermentation.

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[Received, Oct. 13, 1954]

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# 3. *Studies on Lactic Acid Fermentation.*

## *Part 2. Observations on the Mode of Fermentation.*

By Hideo KATAGIRI and Kazutami IMAI

Fermentation of glucouse, gluconate, and xylose under statical and aerobical conditions was carried out by hetero- and homofermenters, and series of fermentation formulas were proposed.

No noticeable difference between homo- and hetero-fermenters was pointed out, except the mode of statical decomposition of glucose which is already known. This well known difference was considered to be due to different types of metabolism during the early stage; glycolysis of the hetero-fermeter would proceed via Warburg-Dickens' scheme instead of Embden-Meyerhof's route, and a scheme of fermentation of lactic acid bacteria was proposed.

### *Experimental and Results*

DeMoss et al.,[1] showed that hetero type lactic acid bacteria possessed no aldolase and deduced that the mechanism of fermentation would not proceed via the Embden-Meyerhof scheme.

The facts that such an organism decomposes pentose to C2 plus C3 compounds (Rappoport et al.,[2]) and that carbon dioxide is derived from the C1 radical of the glucose molecule upon its fermentation,[3] support the other theory of fermentation which disagrees with Embden-Meyerhofs scheme.

In the present paper, experiments were carried out with the following medium, containing: 1.5% yeast extract, 0.5% peptone, 0.5% $KH_2PO_4$, 0.02% $MnSO_4$, 0.04% $MgSO_4$, 0.10% NaCl, 5% $CaCO_3$, and 2∼3% sugar or gluconate, in order to investigate the mode of fermentation of glucose, gluconate, and xylose by lactic acid bacteria.

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[1] D. De Moss, R.C. Bard and I.C. Gunsalus, *J. Bact.*, **62**, 499 (1951).

[2] D.A. Rappoport, H.A. Barker and W.Z. Hassid, *Arch. Biochem. and Biophys.*, **31**, 326 (1951).

Aerobic conditions were brought about by shaking culture and periods of fermentation were four days throughout the experiments.

Acetoin, lactic and acetic acids were determined by the methods described in the previous paper (part 1). Sugar and gluconic acid were determined by the methods of Bertrand and Sumiki[4] respectively. Ethanol was determined by Kolthoff's method.[5]

i) Statical fermentation of glucose

It has been well known that homo- and hetero-fermenters ferments glucose according to the following equations:

$$\text{Homo-fermenter}\quad C_6H_{12}O_6 \longrightarrow 2\,CH_3\cdot CHOH\cdot COOH \qquad (1)$$

$$\text{Hetero-fermenter}\quad C_6H_{12}O_6 \longrightarrow CH_3\cdot CHOH\cdot COOH + C_2H_5OH + CO_2 \qquad (2)$$

As was shown in Table I, the experimental results coincide with the theoretical values.

Table I

Static Fermentation of Glucose

| | Heterofermenter | | Homofermenter | |
|---|---|---|---|---|
| | Found | Theor. Equ. (2) | Found | Theor. Equ. (1) |
| Initial concentration of glucose(%) | 3.0 | | 3.0 | |
| Glucose consumed (%) | 85.0 | | 81.0 | |
| Lactic acid formed (% on consumed glucose) | 50.0 | 50.0 | 95.0 | 100 |
| Alcohol formed (% on consumed glucose) | 28.8 | 25.5 | — | 0 |
| Carbon dioxide formed (% on consumed glucose) | 20.0 | 24.4 | — | 0 |
| Acetoin formation | — | | — | |

Heterofermenter ......*L. fermentum* β; Homoferementer......*L. plantarum*

ii) Aerobic fermentation of glucose

Using hetero- and homo-fermenters, experiments on the fermentation of glucose by shaking culture were carried out and the results are shown in Table II.

From these results, it is found that in the case of hetero-fermenters, glucose is fermented according to the following equation under aerobic conditions.

$$C_6H_{12}O_6 \xrightarrow{+2O} CH_3\cdot COOH + CH_3\cdot CHOH\cdot COOH + CO_2 + H_2O \quad (3)$$

That is to say, under aerobic conditions, almost all the C2 compound is acetate, while under anaerobic condition alcohol is only one compound.

Using the method of paper partition chromatography after Kennedy and Barker[6] acetate was confirmed to be all of the volatile acid, but analytical data showed a slightly smaller yield of lactic acid and a little higher yield of acetic acid when they were compared with theoretical values calculated from equation (3). For this reason, it is considered that this organism has pyruvic oxidase as was mentioned previously (see Part I) and its action is more highly accelerated aerobically than anaerobically, since a small part of

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[3] M. Gibbs and R.D. DeMoss, Fed. Proc., **10**, 189 (1951).

[4] Y. Sumiki, *J. Agri. Chem. Soc. Japan*, **6**, 19 (1930).

[5] L.M. Kolthoff, *Die Massanalyse* (Zweiter Teil) 329 (1928).

[6] E.P. Kennedy and H A. Barker, *Anal. Chem.*, **23**, 1033 (1951).

the pyruvate formed from glucose would be converted to acetate.

With homofermenters, acetate formation again occurred, although formation of acetate is much less than with heterofermenters since the molar ratio of C2 compound (acetate) to C3 compound (lactate) is less that I.

Table II

Aerobic Fermentation of Glucose

| | Heterofermenter | | Homofermenter |
|---|---|---|---|
| | Found | Theor. Equ. (3) | Found |
| Initial concentration of glucose (%) | 2.88 | | 2.74 |
| Glucose consumed (%) | 100 | | 100 |
| Lactic acid formed (% on consumed glucose) | 40.0 | 50.0 | 67.3 |
| Alcohol formed (% on consumed glucose) | 1.4 | 0 | Trace |
| Acetic acid formed (% on consumed glucose) | 37.4 | 33.0 | 14.2 |
| Acetoin formation | − | | + |

Heterofermenter......*L. fermentum β*; Homofermenter......*L. plantarum*

Because acetate is produed by heterofermenters, it was suggested that alcohol formed after equation (2) was oxidized to acetate under aerobic conditions. In order to clarify this point of view, experiments on similar aerobic fermentations using alcohol previously added to the glucose medium were carried out, and the results are shown in Table III.

It is obvious from the results that the addition of alcohol does not change the yield of acetate; this means that added alcohol is never oxidized to acetate. Therefore, it can hardly be said that acetate produced under aerobic conditions is derived from alcohol which is previously formed after equation (2).

Table III

Aerobic Fermentation in Alcohol Added Glucose Medium by Heterofermenter (*L. fermentum β*)

| Addition of alcohol (g/100 ml) | 0 | 1.88 |
|---|---|---|
| Initial concentration of glucose (%) | 3.40 | 3.40 |
| Glucose consumed (%) | 94.1 | 92.7 |
| Lactic acid formed (% on consumed glucose) | 42.0 | 42.1 |
| Alcohol formed (% on consumed glucose) | 1.6 | 0 |
| Acetic acid formed (% on consumed glucose) | 32.2 | 32.3 |

iii) Statical fermentation of gluconate

Using Ca-gluconate as the substrate, experiments on statical fermentation by homo- and hetero-fermenters were carried out. The results are shown in Table IV in which absolutely the same results were obtained with both homo- and hetero-fermenters. From these results, we deduce the metabolic equation of gluconate for homo- and hetero-fermenters as follows ;

$$2\,C_6H_{12}O_7 \rightarrow 2\,CH_3\cdot CHOH\cdot COOH+CH_3\cdot COOH+C_2H_5OH+2\,CO_2+H_2O \quad (4)$$

Table IV

Static Fermentation of Gluconate

| | Heterofermenter | | Homofermenter | |
|---|---|---|---|---|
| | Found | Theor. Equ. (4) | Found | Theor. Equ. (4) |
| Initial concentration of gluconic acid (%) | 2.71 | | 2.75 | |
| Gluconic acid consumed (%) | 50.9 | | 81.0 | |
| Lactic acid formed (% on consumed gluconic acid) | 52.1 | 45.9 | 47.6 | 45.9 |
| Alcohol formed (% on consumed gluconic acid) | 13.4 | 11.7 | 10.1 | 11.7 |
| Acetic acid formed (% on consumed gluconic acid) | 18.0 | 15.3 | 19.7 | 15.3 |
| Carbon dioxide formed (% on consumed gluconic acid) | 24.8 | 22.4 | | 22.4 |
| Acetoin formation | – | | – | |

iv) Aerobic fermentation of gluconate

Using the sa memedium as mentioned above (Experiment iii), experiments of shaking cultures with homo- and hetero-fermenters were carried out.

These results are shown in Table V. From these results, we deduce the metabolic equation of gluconate for heterofermenters under aerobic condition as follows;

$$C_6H_{12}O_7 \xrightarrow{+O} CH_3\cdot CHOH\cdot COOH+CH_3\cdot COOH+CO_2+H_2O \quad (5)$$

In the case of homofermenters, the products were again found to be lactate and acetate, but the former yield was less and the latter was more than the theoretical values calculated from equation (5). The reason is not clear, but it may be attributable to the pyruvic oxidase of the homofermenter whose activity is superior to that of heterofermenter.

Table V

Aerobic Fermentation of Gluconate

| | Heterofermenter | | Homofermenter |
|---|---|---|---|
| | Found | Theor. Equ. (5) | Found |
| Initial concentration of gluconic acid(%) | 2.71 | | 2.84 |
| Gluconic acid consumed (%) | 85.9 | | 81.7 |
| Lactic acid formed (% on consumed gluconic acid) | 41.5 | 45.9 | 31.0 |
| Alcohol formed (% on consumed gluconic acid) | 0 | 0 | 0 |
| Acetic acid formed (% on consumed gluconic acid) | 37.3 | 30.6 | 48.3 |
| Acetoin formation | – | | – |

v) Statical fermentation of xylose

It was already pointed out that both homo- and hetero-fermenrers ferment pentose in the same manner as is illustrated in the following equation:

$$C_5H_{10}O_5 \longrightarrow CH_3\cdot COOH+CH_3\cdot CHOH\cdot COOH \qquad (6)$$

Our results, as shown in Table VI, were found to coincide with this equation.

Table VI

Static Fermentation of Xylose

| | Heteroformenter Found | Homofermenter Found | Theor. Equ. (6) |
|---|---|---|---|
| Lactic acid formed (% on consumed xylose) | 57.1 | 62.7 | 60.0 |
| Acetic acid formed (% on consumed xylose) | 39.4 | 40.5 | 40.0 |
| Alcohol formed (% on consumed xylose) | Trace | Trace | 0 |
| Acetoin formation | — | — | |

Heterofermenter……*L. fermentum β*; Homofermenter……*L. xylosus*

vi) Aerobic fermentation of xylose

The analytical results of these fermented liquids by homo- and hetero-fermenters, as is shown in Table VII, are found to be similar to those of statical fermentation. Hence the mode of pentose fermentation coincides with equation (6) irrespective of fermenting conditions or type of microorganism.

Table VII

Aerobic Fermentation of xylose

| | Heterofermenter Found | Homofermenter Found | Theor. Equ. (6) |
|---|---|---|---|
| Initial concentration of xylose (%) | 2.76 | 2.45 | |
| Xylose consumed (%) | 50.1 | 69.4 | |
| Lactic acid formed (% on consumed xylose) | 59.9 | 55.0 | 60.0 |
| Acetic acid formed (% on consumed xylose) | 41.2 | 33.6 | 40.0 |
| Alcohol formed (% on consumed xylose) | 0 | 0 | 0 |
| Acetoin formation | + | + | |

## *Discussion*

As previously mentioned, the mechanism of glycolysis of hetero-type lactic acid bacteria does nos follow Embden-Meyerhof's scheme, so that we must seek another metabolic route for it.

The fermentation formulas of glucose, gluconate, and xylose by hetero-type lactic acid bacteria under static and aerobic conditions which were proposed by us, summarized as follows:

$$\text{Glucose}\begin{cases}\text{Static; } C_6H_{12}O_6 \longrightarrow CH_3\cdot CHOH\cdot COOH+CH_3\cdot CH_2OH+CO_2 & (2)\\ \text{Aerobic; } C_6H_{12}O_6 \xrightarrow{+2O} CH_3\cdot CHOH\cdot COOH+CH_3\cdot COOH+CO_2+H_2O & (3)\end{cases}$$

$$\text{Gluconate}\begin{cases}\text{Static; } 2\,C_6H_{12}O_7 \longrightarrow 2\,CH_3\cdot CHOH\cdot COOH+CH_3\cdot CH_2OH+CH_3\cdot COOH+2CO_2+H_2O & (4)\\ \text{Aerobic; } C_6H_{12}O_7 \xrightarrow{+O} CH_3\cdot CHOH\cdot COOH+CH_3\cdot COOH+CO_2+H_2O & (5)\end{cases}$$

$$\text{Xylose}\begin{cases}\text{Static; } C_5H_{10}O_5 \longrightarrow CH_3\cdot CHOH\cdot COOH+CH_3\cdot COOH & (6)\\ \text{Aerobic; } C_5H_{10}O_5 \longrightarrow CH_3\cdot CHOH\cdot COOH+CH_3\cdot COOH & (6)\end{cases}$$

In the case of static fermentation of glucose, C2 compounds formed consists almost entirely of alcohol, and its molecular ratio to the C3 compound (latic acid) is 1.0. On the other hand, with gluconate which is an oxidized form of glucose, alcohol and acetic acid are formed in equimolecular proportions by static fermentation, but the molecular ratio of C2 compounds to the C3 compound is again 1.0 as was observed with glucose. While in static fermentation of xylose, the C2 compound formed consists entirely of acetate, and its molecular ratio to the C3 compound is still 1.0. Under aerobic conditions, the C2 compound formed from glucose or gluconate is always acetic acid, and alcohol was never detected. Moreover the molecular ratio of the C2 compound (acetic acid) to the C3 compoud (lactic acid) is also 1.0. In the case of xylose, the products of aerobic fermentation are identical with those of anaerobic fermentation, that is the C2 compound formed is acetate and its molecular ratio to lactate is 1.0, and evolution of carbon dioxide was never detected. From these results, it is strongly suggested that the mode of glycolysis by heterofermenters would proceed via Warburg-Dickens' scheme (glucose⟶gluconic acid⟶C5 compound⟶) instead of Embden-Meyerhof's route. It is interesting to point out the remarkable difference between homo- and hetero-fermenters in static decomposition of glucose, namely the former produces lactic acid as the sole end product, and the latter produces carbon dioxide and ethanol in addition to lactic acid, but when gluconate or xylose is employed as the substrate, no difference in the mode of static metabolism is observed between homo- and heterofermenters, as illustrated in the following equations with homo-fermenters;

$$\text{Glucose}\begin{cases}\text{Static; } C_6H_{12}O_6 \longrightarrow 2\,CH_3\cdot CHOH\cdot COOH & (1)\\ \text{Aerobic; } C_6H_{12}O_6 \xrightarrow{+2O} CH_3\cdot CHOH\cdot COOH+CH_3\cdot COOH+CO_2+H_2O & (3)\end{cases}$$

$$\text{Gluconate}\begin{cases}\text{Static; } 2\,C_6H_{12}O_7 \longrightarrow 2\,CH_3\cdot CHOH\cdot COOH+CH_3\cdot CH_2OH\\ \qquad\qquad +CH_3\cdot COOH+2\,CO_2+H_2O & (4)\\ \text{Aerobic; } C_6H_{12}O_7 \xrightarrow{+O} CH_3CHOH\cdot COOH+CH_3\cdot COOH+CO_2+H_2O & (5)\end{cases}$$

$$\text{Xylose}\begin{cases}\text{Static; } C_5H_{10}O_5 \longrightarrow CH_3\cdot CHOH\cdot COOH+CH_3\cdot COOH & (6)\\ \text{Aerobic; } C_5H_{10}O_5 \longrightarrow CH_3\cdot CHOH\cdot COOH+CH_3\cdot COOH & (6)\end{cases}$$

Therefore the difference in static decomposition of glucose between homo- and hetero-fermenters is considered to be attributable to the route; glucose ⟶gluconic acid, which is the main road only for heterofermenters. And we deduce that the source of alcohol is not pyruvate but an unidentified C2 compound which is derived from a C5 compound (presumably pentose).

Ethyl alcohol is produced when hydrogen is transported to this C2 compound, and if hydrogen transportation does not occur, the ethyl alcohol is converted to acetic acid. Furthermore we think that such hydrogen is donated from glucose or gluconate by the proper dehydrogenase system. In the case of aerobic fermentation this coupling may be blocked by oxygen, so that the C2 compound will not be reduced to ethanol, but converted to acetate.

From these investigations, we present the following scheme (Fig. 1) for the mechanism of hetero-type lactic acid fermentation.

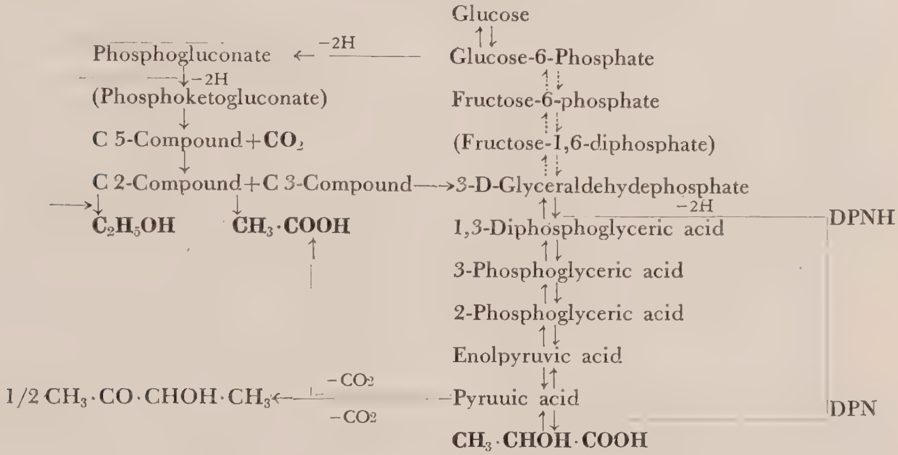

Fig. 1. Scheme of Lactic Acid Fermentation

The route shown by the dotted line is the main path of the homofermenter, while there is no indication that the heterofermenter follows this path.

With heterofermenter, we detected acetoin from the culture of xylose medium under aerobic conditions. Acetoin formation is again detected with homofermenters from the cultures of both glucose and xylose media under aerobic conditions. These facts indicate that the C3 compound derived from the C5 compound (xylose) will be converted to pyruvate, and then acetoin is produced from certain parts of the pyruvate as was already verified in the previous paper (Part 1).

In our laboratory Katagiri and Kitahara[7] studied the mechanism of mannitol fermentation by heterofermenters, and found that if the yield of mannitol was less than the theoretical value calculated from the following equation, there occurred the formation of ethyl alcohol which was verified not to be derived from mannitol, but from fructose.

$$3\,C_6H_{12}O_6 + H_2O \rightarrow 2\,C_6H_{14}O_6 + CH_3 \cdot CHOH \cdot COOH + CH_3 \cdot COOH + CO_2$$

In this case it was shown that the more mannitol formed, the more acetic acid and the less alcohol was formed. This fact is explained by the assumption that if hydrogen is derived from the decomposition of fructose, is would be entirely transported to the other fructose molecule in producing mannitol, and there would appear only acetate among the C2 compounds as reduction of the C2 compound to alcohol could not take place.

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[Recieved, Oct. 13, 1954]

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[7] H. Katagiri and K. Kitahara, *Nihon Gakujutsu Kyokai Hokoku*, **16**, 548, (1942).

# 4. *Report 1. Studies on the α-Amylase Destroying Enzyme.*

## *Part 1. Occurrence and Some Properties of the Enzyme.**

By Takehiko YAMAMOTO

A certain species of bacteria secretes an enzyme which causes deactivation of several α-amylases. This enzyme seems to be an adaptive enzyme formed against these α-amylases. Enzymatical studies show that the enzyme is sensitive to heat, acid, and alkali; the optimum pH of its activity is at about 5.2; it requires phosphates as a cofactor in order to display its enzymatic action, and as the enzyme deactivates α-amylases, titratable acid is formed.

In the culture experiments of α-amylase producing bacteria, it was often experienced that contamination by a certain species of bacteria brings about a rapid decrease of α-amylase activity without causing any appreciable change in the reaction of the media. This fact was of much interest, because most of the microorganisms found in the contaminated culture do not cause such a rapid decrease of amylase activity, except when they form acids in quantities leading to the denaturation of the enzyme. It has been known that the active α-amylases are not inactivated by proteases such as trypsin or papain[1]. From these facts, it was supposed that such a rapid decrease of amylase activity caused by contamination might be due to some specific substance produced by the organism of such a nature as to cause the destruction of α-amylase. The cultivation of the bacteria was therefore undertaken, and the influence of the culture broth upon the α-amylase activity was investigated using mainly crystalline bacterial α-amylase.* The experimental results led to the conclusion that the factor destroying α-amylase is an enzyme. In the present paper, some factors affecting the formation of the enzyme and its enzymatic properties will be described.

### *Experimental*

The bacteriological studies showed that the isolated bacteria grow facultatively and do not form spores. They grow on bouillon or on analogous media. On the semi-synthetic medium, recognized as comparatively suitable for growth of these bacteria, which

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* This investigation was supported in part by a Research Grant from the Education Department.

[1] S. Akabori, S. Hayashi and R. Kasimoto, *J. Chem. Soc. Japan*, **61**, 1035 (1940); S. Akabori and K. Okahara, *ibid*, **12**, 55 (1937).

* Two types of bacterial α-amylase (2) have been obtained in crystalline form, one is amyloclastic α-amylase (3) from *Bac. amyloliquefaciens* Fukumoto (4), and the other is saccharogenic α-amylase (5, 6) from a strain of *Bac. subtilis*. In the present paper, bacterial α-amylase represents only the former, unless otherwise specified.

[2] J. Fukumoto, T. Yamamoto and K. Ichikawa, *Symposia on Enz. Chem.* (Japan), **8**, 40 (1953).

[3] B. Hagiwara, *Proc. Jap. Acad.*, **27**, 346(1951).

[4] J. Fukumoto, *J. Agr. Chem. Soc.* (Japan), **19**, 487, 634, 689, 789, 853 (1944), **20**, 23, 121,309(1944).

[5] J. Fukumoto, T. Yamamoto and K. Ichikawa, *Proc. Jap. Acad.*, **27**, 252 (1951).

[6] H. Fuwa and Z. Nikuni, *J. Agr. Chem. Soc.* (Japan), **26**, 154 (1952).

contained starch hydrolized by bacterial saccharogenic $\alpha$-amylase, 3.0%; polypeptone, 0.3%; sodium citrate, 0.1%; ammonium phosphate, 0.3%; magnesium sulfate and potassium chloride, 0.01%, in tap water and the pH adjusted to 7.0, the bacteria multiply evolving a trace of gas which is accompanied by gradual lowering of the pH of the medium to about 5.0, where the multiplication completely ceases.[*]

However, this culture broth shows no effect on $\alpha$-amylase activity. On the other hand, growth of the bacteria is somewhat more vigorous in media to which bacterial $\alpha$-amylase has been supplied, and it was found that the culture filtrate of such media deactivates the $\alpha$-amylase subsequently added remarkably. This seems to show that it is an adaptive enzyme against bacterial $\alpha$-amylase. The relationship between the amount of bacterial $\alpha$-amylase added to the medium and the activity of amylase destruction of the culture filtrate is given in Table I.

Table I

Relationship between the Amount of Bact. $\alpha$-Amylase added to Medium and the Amylase-Destructive Activity of Culture Filtrate.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Amylase added, L.S.V. per cc medium[*] | 0 | 0.2 | 2 | 10 | 20 | 50 | 100 |
| Activity of culture filtrate[**] | 0 | + | 150 | 620 | 780 | 800 | 800 |

[*] The bacteria were grown on the medium containing polypeptone, 0.5%; silk-worm pupa extract, 0.5%; maltose, 3%; NaCl, 0.5%; and amylase indicated in the coloumn, at 36°C for 48 hr.,, 1mg of the of the crystalline amylase shows an activity of 2,000L.S.V.[***] which indicates the amount of potato starch in g liquefied by the amylase in 15 min. It is also possible to liberate 1770 mg of maltose-equivalent sugar from soluble starch in 5 min. at 40°C.

[**] 1cc of culture filtrate, 1 cc of amylase (1,500L S.V.) and 1cc of 1/15 mol phosphate were incubated at 36°C for 16hr. The number represents the decrease of amylase activity.

For the enzyme formation, the heat-denatured bacterial $\alpha$-amylase was found to be effective to the same extent as the active amylase, but acid-denatured amylase showed no effect. Furthermore, taka-, malt $\alpha$-, and swine pancreatic-amylase were also effective, but bacterial saccharogenic $\alpha$-amylase was entirely negative. Proteins such as milk-casein and egg-albumin, or soya-been-cake meal were also ineffective.

It was possible to purify the enzyme to some extent according to the following method. The bacteria were grown at 36.5°C for sixty hours on the semi-synthetic medium as described previously to which about 12 $\mu$g of crystalline bacterial $\alpha$-amylase per cc of medium has been added. The culture filtrate (3 l, pH 5.2) was treated with lead acetate (200cc of a 25% solution). To this filtrate, ammonium sulfate (1,300 g) was added and it was allowed to stand for several hours. Thereafter, additional ammonium sulfate (530 g) was added to the supernatant solution. After standing overnight in the cold, the flocculent precipitate was separated by centrifuugation and dissolved in distilled water followed by filtration. Salting out with ammonium sulfate was again carried out on this filtrate. The resulting precipitate was dissolved in a small quantity of distilled water, and it was dialysed against distilled water at 6°C for four days. An equal volume of acetone was added to the filtrate of the dialysed solution and then centrifuged. To the supernatant solution, another equal volume of acetone was added. A small amount of precipitate was formed wherein enzyme activity was found (total nitrogen, 0.6 mg; activity yield, about 17%). This partially purified preparation produced a faint but distinct flocculent precipitate upon addition of trichloroacetic acid and gave a positive ninhydrine reaction after hydrolysis.

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[*] Morphology and Physiology of the bacteria is to be reported in another paper in details.

[***] C.J. Linter und P. Sollied, *Z.ges.Brau.*, **26**, 103 (1903).

However, the enzyme thus purified showed activity only in the presence of phosphates. As can be seen in Fig. 1, the activity increases with increase in phosphate concentration up to 1/40 mol, but inversely decreases with further increase in the phosphate concentration. In the presence of 1/40 mol phosphate, the partially purified enzyme described above showed an activity (per unit of protein nitrogen) twenty times greater than that of the original culture broth. The preparation showed no proteolytic activity by the formol method when milk-casein or gelatin was used as substrate, either in the presence or abscence of phosphate.

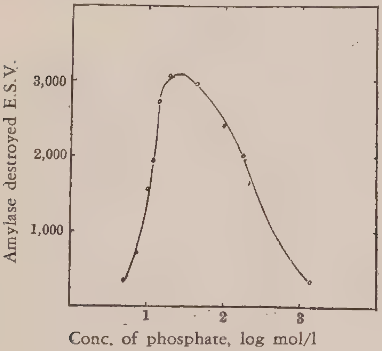

Fig. 1. Effect of various concentration of phosphate on the activity of destroying enzyme.

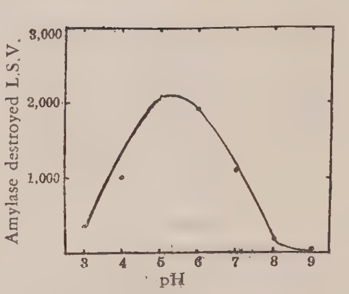

Fig. 2a. Changes of activity after treating at various pH values; 30 min., 37°C.

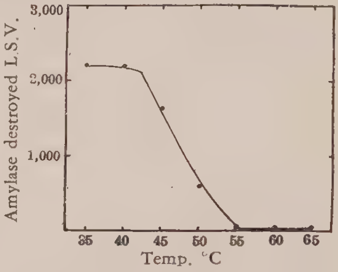

Fig. 2b: Changes of activity after treating at various temperatures; 10 min., pH 5.0.

The enzyme is sensitive, as will be seen in Fig. 2a and 2b, i.e., its activity is completely lost by heating at 50°C for ten minutes and deteriorates easily at pH values lower than 4.0 or at pH values higher than 7.0.

Effects of several factors upon the enzyme activity are shown in Figs. 3a, 3b and 4. The optimum pH of the enzyme action lies at about 5.2, and the optimum temperature is found in the vicinity of 36°C in a twenty-four

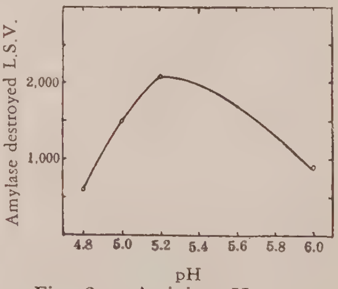

Fig. 3a. Activity-pH curve; 36°C, 24 hr..

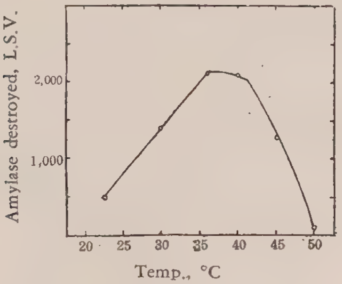

Fig. 3b. Activity-temp. curve; pH 5.2, 24 hr..

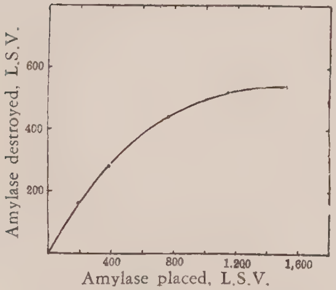

Fig. 4. Relationship between the deterioration rate of activity and the amount of bact. α-amylase; 37°C, 14hr.

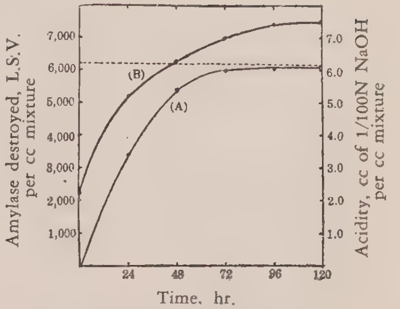

Fig. 5. Deterioration of activity and acid formation of bact. α-amylase by the destroying enzyme.
(A) deterioration of the activity;
(B) acidity. Amylase, 10.9 mg (74 mg as protein) and destroing enzyme, 0.048 mg, as protein N in 1/80 mol phosphate sol., total volume 24 cc, pH 5.2, 36.5°C; the broken line indicates the initial amylase activity.

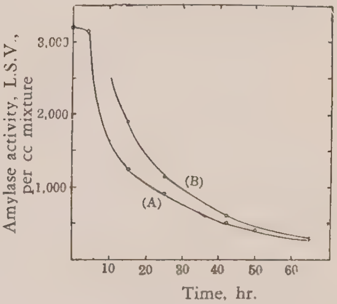

Fig. 6. Effect of dialysis on the deteriorating bact. α-amylase by the destroying enzyme.
(A) values of intact mixture;
(B) values of dialysed sol.. Amylase, 5.17 mg and destroying enzyme, about 0.01 mg, as protein N in 1/40 mol phosphate sol., total volume at the reaction, 21 cc; pH 5.2, 37°C.

hours' test. An experiment on the relationship between the concentration of substrate (the concentration of bacterial α-amylase) and the rate of reaction (the rate of deactivation of the amylase) indicated, as shown in Fig. 4, that the deactivation rate increases to some extent with increase in amylase concentration.

It was found that addition of the enzyme to the solution of crystalline bacterial α-amylase brings about a slight turbidity within three to six hours, and generally, the deactivation of amylase activity becomes appreciable thereafter. From the data shown in Fig. 5 and 6, it is evident that the deterioration of amylase activity roughly follows the equation of a first-order reaction. Fig. 5 also shows that during the reaction titratable acid is produced in considerable quantities almost proportional to the deactivation of the amylase. However, the change in pH was not more than 0.2 units during the reaction. In the later stage of the reaction the mixture permitted formol titration, and paper-chromatogrames revealed that liberation of free amino acids and peptides occurred.

Experiments showed that the deterioration curve of amylase activity of the reaction mixture differs somewhat when the amylase activity was eveluated before and after the mixture had been dialysed using a cellophane bag against distilled water, the pH of which had been adjusted to 7.0 with a dilute solution of sodium hydroxide. As can be seen in Fig. 6, dialysis causes some recovery of amylase activity which had fallen, to a certain extent, but this is evident only at the early stage of the reaction.

Table II

Effect of Destroying Enzyme on the Activity of Several Amylases.

| | pH of mixture | Amylase activity, u. per cc after* 0 | 20hr. | Deactivation; % |
|---|---|---|---|---|
| Bacterial $\alpha$-amylase** | 5.2 | 1125 | 345 | 69.3 |
| Bact. sachrogenic $\alpha$-Amylase** | 5.2 | 980 | 980 | 0 |
| Malt $\alpha$-Amylase** | 5.2 | 1000 | 352 | 64.8 |
| Malt $\beta$-Amylase*** | 5.2 | 190 | 190 | 0 |
| Taka $\alpha$-Amylase** | 5.2 | 1260 | 413 | 67.2 |
| Pancreatic $\alpha$-Amylase** | 6.0 | 790 | 366 | 53.7 |
| Gluc-Amylase*** | 5.0 | 130 | 130 | 0 |

* The reaction mixture (final conc. of phosphate, 1/40 mol) was incubated at 36°C for 20 hr.

** $\alpha$-Amylase activity was determined by the iodine color method where one unit was defined as the activity to render 10cc of 1% soluble starch achromatized in 60 min. at 40°C.

*** The activity of these amylases were determined by the saccharification test, the values representing the number of glucosidic bonds, $\mu$mol, hydrolized by the enzyme for 5min. at 40°C

The enzyme destroys not only bacterial $\alpha$-amylase, but also taka-, malt $\alpha$-, and swine pancreatic-amylase. However, as given in Table II, it shows no adverse effect on bacterial saccharogenic $\alpha$-amylase, and also on $\beta$-amylase of malt or gluc-amylase[7]. Table II also indicates that the enzyme can cause approximately the same degree of deactivation within a fixed time when added to bacterial $\alpha$-, taka-, malt $\alpha$-, and swine pancreatic-amylase of an almost equal level of the activity.

### *Discussion*

That the active substance capable of causing the deactivation of $\alpha$-amylase is an enzyme may be sufficiently substantiated by the facts that it is a sensitive substance of protein nature and its activity is easily influenced by environmental factors similar to many enzymes, and that the deterioration of $\alpha$-amylase by the substance progresses with time. If it were merely an amylase inhibitor[8], it would only show a definite extent of inactivation of $\alpha$-amylase, irrespective of time.

It is noteworthy that the enzyme attacks bacterial $\alpha$-, taka-, malt $\alpha$-, and swine pancreatic-amylase but not bacterial saccharogenic $\alpha$-amylase, and that the enzyme formation occurs only in the presence of the former group of $\alpha$-amylases and not in the presence of the latter. This fact lead to the supposition that there is a critical site sus-

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[7] L.L. Phillips and M.L. Caldwell, *J. Am. Chem. Soc.*, **73**, 3559 (1951); J. Fukumoto, Y. Tsujisaka and K. Minami, *Science and Industry* (Japan), **28**, 92 (1954).

[8] E. Kneen and R.M. Sandstedt, *J. Am. Chem. Soc.*, **65**, 1247 (1943); B. S. Miller and E. Kneen, *Arch. Biochem.*, **15**, 251 (1947).

Table III

Effect of Several Proteases on the Activity of Bact. α-Amylase.

| | pH | Amylase activity per cc after<br>0 | 24hr. | 48hr. |
|---|---|---|---|---|
| Crystalline trypsin | 7.6 | 168 | 168 | 168 |
| Crystalline chymotrypsin | 7.4 | 133 | 133 | 133 |
| Pepsin | 5.2 | 200 | 200 | 200 |
| Bact. protease | 6.6 | 220 | 220 | 220 |
| Proteases of pancreas | 7.6 | 410 | 410 | 410 |
| Destroying enzyme | 5.2 | 210 | 24 | 0.5 |
| No addition | 5.0 | 220 | 220 | 220 |

ceptible to the enzyme in these α-amylase proteins, which seems to be essential to maintain the amylase activity.

However, no decisive experimental results has yet been obtained to clarify the mechanism of the destruction of α-amylase by the enzyme. It may be supposed that a proteolytic enzyme deactivates native proteins such as enzymes for the reason that it tends to shift the equillibrium between the native and denatured form of the protein as a result of incessant attack on the denatured form. But, actually, as given in Table III, no appreciable deactivation of bacterial α-amylase was found by protease, such as trypsin, chymotrypsin or bacterial protease, and even by a crude protease preparation from swine pancreas, although Fischer and Bernfeld[9] reported that the instability of an impure solution of pancreatic amylase is due to the presence of a specific protease.

Experiments showed that free amino acids and peptides were present in the reaction mixture at the end of deactivation of bacterial α-amylase by the enzyme, indicating that hydrolysis of peptide bonds of the amylase protein occurred. However, it is not considered that the deactivation results directly from the hydrolysis of amylase protein. Probably, this liberation of amino acids and peptides is not related to the deactivation of amylase, because although there is a possibility of contamination of certain type of protease, it is known that the protease shows no effect at least on bacterial α-amylase activity itself. This subject will be taken up in a subsequent paper.

On the other hand, the fact that the enzyme necessitates phosphates in order to display its activity and that deactivation of bacterial α-amylase is accompanied by an increase of titration acidity may present some clues to the mechanism of deactivation, quite apart from the problems of whether they are the principal cause or subordinate phenomena. It may also lead to clarify, to a certain extent, the mechanism that all these α-amylases attacked by the enzyme are stabilized by the presence of calcium ions, and that all the crystals of these α-amylases ever obtained contain calcium[10,11,12,13].

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[9] E.H. Fischer and P. Bernfeld, *Helv.Chim.Acta*, **31**, 1839 (1948).

[10] B. Hagiwara, *Symposia on Enz.Chem.* (Japan), **7**, 105 (1952).

[11] Ed. H. Fischer et R. de Montomollin, *Helv. Chim. Acta*, **34**, 1987 (1951); S. Akabori, T. Ikenaka and B. Hagiwara, *Symposia on Enz. Chem.* (Japan), **7**, 107 (1952).

[12] S. Schwimmer and A.K. Balls, *J.Biol.Chem.*, **179**, 1063 (1949).

[13] M.L. Caldwell, M. Adams, J. Tung and G. Toralballa, *J. Am. Chem. Soc.*, **74**, 4033 (1952).

The nomenclature of enzyme should be dealt with on the basis of linkage specificity, thus, the enzyme will be referred to only as an "α-amylase destroying enzyme," while its mechanism has not yet been clarified.

*Acknowledgements*

The author wishes to thank Prof. Dr. I. Yamasaki of Kyushu University, for his helpful advice, and to Prof. Dr. J. Fukumoto of Osaka City University, for his instructive suggestions throughout these works. The author also wishes to thank Mr. G. Chiba, for his assistance in the analysis.

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## 5. Über die Oxydation des Lysozyms mit Perjodat

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Lysozyme was oxidized with sodium meta-periodate, and the following results were obtained from investigations of the process of oxidation and the changes of lytic activity and absorption spectrum. The lytic activity decreases during oxidation. Lysozyme oxidized under moderate conditions was obtained as crystals with 60% of the original activity. The decrease in lytic activity has a linear relationship to the lowering of the optical density at 280.5 mμ. This lowering being caused chiefly by the decomposition of tryptophan, it may be concluded that tryptophan residue seems to play an important role in lytic activity.

Meyer et al. haben berichtet, dass das Lysozym durch Peroxyd, Jod u.s.f. inaktiviert, und durch die reduzierenden Agenzien wie Schwefelwasserstoff oder Blausäure wieder aktiviert wird[1,2]. Neuerdings oxydierten Kertësz et al. mittels des Systems, Polyphenoloxydase und *o*-Dioxyphenols, Lysozym[3,4]. Die Tyrosyl-Gruppe des Lysozyms erlitt durch dieses System eine umkehrbare Oxydation und die dabei entstandene, rote Oxyprobe hatte die Hälfte der originalen Aktivität[5]. Ihre Aktivität jedoch erschien wieder durch die

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[1] K. Meyer und E. Hahnel, *J. Biol. Chem.*, **163**, 723 (1946).
K. Meyer, R. Thompson, J.W. Palmer und D. Khorazo, *J. Biol. Chem.* **113**, 303 (1936).

[2] G. Alderton, H.L. Fevold und H.O. Lightbody, *Federation Proc.*, **5**, 119 (1946).

[3] D. Kertész und M. Teti, *Compt. rend. Soc. Biol.*, **144**, 923 (1950).

[4] M. Teti, *Riv. ist. sieroterap. Ital.* **26**, 185 (1951); zitiert aus *C. A.*, **46**, 4032 (1952).

[5] D. Kertész und P. Caselli, *Bull. Soc. Chim. biol.*, **32**, 583 (1950).

Reduktion mit Natriumhyposulfit.

Fraenkel-Conrat[6] folgerte hauptsächlich aus seinem Experimente von der Jod-Oxydation, dass es sich hier als die unentbehrliche Gruppe für lytische Aktivität nicht, wie man annehmen könnte, um die Phenol- oder Thiol-Gruppe, sondern um die Gesamtheit von Amino-, Carboxyl-, Amide-, Guanidyl, aliphatischer Hydroxyl-Gruppe, und insbesondere Imidazol von Histidin handelt. Also stimmen nicht alle Folgerungen überein.

Im Rahmen unserer Versuche zur Oxydation von Eiweissstoffen haben wir uns auch die Frage vorgelegt, ob sich nicht aus solchen Versuchen ein Verfahren zur Aufklärung der unentbehrlichen Lage für lytische Aktivität herleiten liesse.

In unseren Versuchen wurde Lysozym wie andere Eiweissstoffe unter verhältnismässig milden Bedingungen oxydiert, ohne zu koagulieren. Das oxydierte Lysozym kristallisierte sich aus dem Reaktionsgemische auch in dem Oxydationsgrade von 2.5 Mol $NaJO_4$/Mol Lysozym. Die lytische Aktivität nahm infolge der Oxydation von Perjodat ab, und liess sich durch die Reduktion mittels des Schwefelwasserstoffes oder des Cysteins nicht wiederherstellen. Nach der Oxydation blieb Cystin-Gehalt fast unverändert und Histidin erlitt fast keine Veränderung. Dass das Tryptophan erhebliche Zersetzung litt, wurde jedoch ermittelt.

Mit der wachsenden Oxydation zeigte aber das UV-Absorptionsspektrum von Lysozym erkennbare Änderung, insbesondere erhebliche Verminderung der Extinktion in dem Maximum von 280.5 mμ[7,8,9].

Wie man an der Figur 1 erkennt, steht die Verminderung der Extinktion in dem Absorptionsmaximum in enger Beziehung, d.h. in der Funktion erster Ordnung mit der Abnahme der lytischen Aktivität. Das Herabsinken von dem Absorptionsgipfel beruht aber hauptsächlich auf der Zersetzung von Tryptophan in den Aminosäuren, die zur Absorption von 280.5mμ beigetragen haben[10]. Daraus lässt sich also schliessen, dass das Tryptophan für die lytische Aktivität von Lysozyme eine wichtige Rolle spielt.

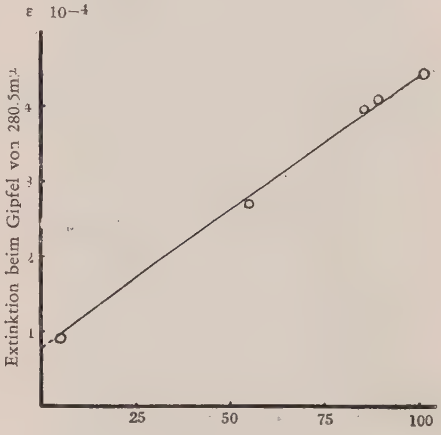

Fig. 1. Verminderung der Extinktion in dem Absorptionsmaximum des Lysozyms und Abnahme der lytischen Aktivität infolge der Oxydation.

Obwohl das Perjodat mit den am Ende stehenden Aminosäuren, Oxyaminosäuren oder besonders Histidine von Lysozyme in der eigentümlichen Weise reagiert haben möchte, so müsste das doch keine Änderung bei 280.5mμ

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6) H. Fraenkel-Conrat, *Arch. Biochem.*, **27**, 109 (1950).

7) C. Fromageot, *Bull. Soc. Chem. biol.*, **30**, 63 (1948).

8) P. Caselli, *Arch. Sci. Biol.*, **33**, 414 (1949).

9) L.R. Wetter und H.F. Deutsch, *J. Biol,Chem.*, **192**, 237 (1951).

10) K. Maekawa und M. Kushibe, *Sci. Rep. Matsuyama Agric. Coll.*, **12**, 33 (1954).

des Spektrums verursachen. Die letzteren mögen also wirklich unentbehrliche Gruppen sein und die Tatsache, dass die Zersetzung von Tryptophan in enger Beziehung mit der Verlierung der Aktivität steht, mag nur scheinbar sein. Von anderen Seiten müssen weite Versuche angestellt werden, um darüber zu entscheiden[10].

## *Beschreibung der Versuche*

### 1. Die Menge des verbrauchten Perjodats durch Lysozym und die Abnahme der lytischen Aktivität.

a) Oxydation :—Lysozym, welches nach der Vorschrift von Aldelton dargestellt[11], mehrfach umkristallisiert und centrifugiert wurde, wurde in 0.1 N Acetate-Puffer von pH 5.4 aufgelöst, um Enzymlösung von 4-5 mg/ccm darzustellen (nach Biuret). Natrium meta-Perjodat wurde aus Wasser umkristallisiert und in demselben Puffer (oder im Wasser) als 0.1 N. Lösung gelöst. 20 ccm dieser Lysozymlösung wurde in 50 ccm Glasstöpselflaschen pipettiert und bis 4-5°C abgekühlt, hierzu wurde 5 ccm der oben genannten Perjodatlösung (4-5°C) zugesetzt, dann wurde diese bei 4-5°C unter gelegentlicher Pipettierung gelassen. Indem man 1 ccm von der ausgezogenen Lösung benutzte, wurde Perjodat-Verbrauch nach der früher mitgeteilten Vorschrift bestimmt[12].

b) Lytische Aktivität:—Die lytische Aktivität von Lysozyme wurde durch eine Modifikation des von Boasson beschriebenen Verfahrens[9] bestimmt. Die Suspension von *Micrococcus lysodeikticus* wurde nämlich mit 0.1N. Phosphat-Puffer von pH 7.3 drei Male gewaschen und centrifugiert. Nachdem die Bakterien in demselben Puffer wieder suspendiert worden waren, wurden sie zwei Minuten lang bei 2,000 r.p.M. centrifugiert, um Klümpchen zu entfernen und beständige Suspension zu erhalten. Diese Suspension wurde mit demselben Puffer derart verdünnt, dass die Durchlässigkeit des Lichtes bei der Anwendung des Photokolorimeters beinahe 25% beträgt, wenn die Messung beim Filter 530m$\mu$ und Kuvettenschichtdicke 1.0 cm durchgeführt wird.

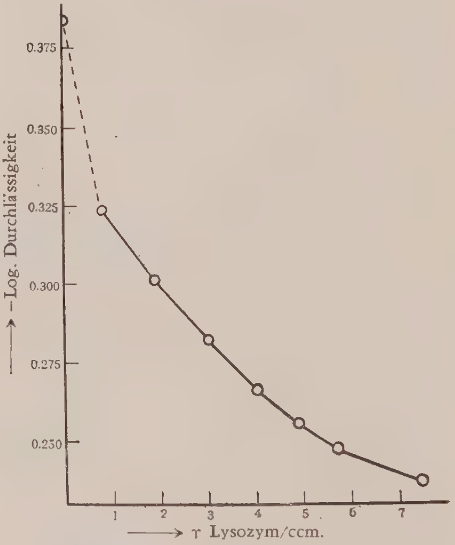

Fig. 2. Beziehung zwischen der Lysozymkonzentration und der Durchlässigkeit von Lichte durch Gemisch des Lysozyms und bakterieller Suspension.

Zu 5 ccm dieser vorbereiteten Suspension wurden 1-3 ccm von Lysozymlösung (1-5 $\gamma$ Lysozym/ccm Lösung) und 2-0 ccm 0.1 N. Phosphat-Puffer von pH 7.3 zugesetzt. Nachdem bei 25°C, 15 Minuten lang gelassen worden war, wurde die Messung der Extinktionswerte ausgeführt. Die Figur 2 zeigt die dabei erhaltenen Beziehungen zwischen der Lysozymkonzentration und der Durchlässigkeit von Lichte.

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[11] G. Alderton und H.L. Fevold, *J. Biol. Chem.*, **164**, 1 (1946).

[12] K. Maekawa und Kushibe, *Bull. Chem. Soc. Japan*, **27**, 277 (1954).

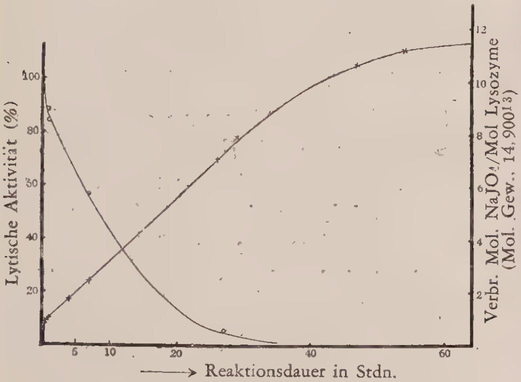

Fig. 3. Perjodat-Verbrauch von Lysozyme (×——×) und die Abnahme der lytischen Aktivität (○——○).

Wenn man die Aktivität von oxydiertem Lysozyme mass, zog man besonders Stickstoff-Gehalt von Lösung in Erwägung. Die einige Male wiederholten Ablesungen stimmten innerhalb des Fehlers von 3-5 % überein. Die Menge des zeitlichen Perjodat-Verbrauchs und die damit begleitete Abnahme der lytischen Aktivität werden in der Figur 3 gezeigt.

Wie man an der Figur 3 erkennt, reduziert Lysozym pro M. bei pH 5.4, 5°C 11 M. von Perjodat (Mol. Gew. des Lysozyms als 14,900[13] annehmend). Das oxydierte Lysozym kristallisierte sich meistenteils in der früheren Stufe aus Reaktionsgemische.

## 2. Darstellung des oxydierten Lysozyms.

Das mehrfach umkristallisierte Lysozym wurde in verd. Essigsäure von

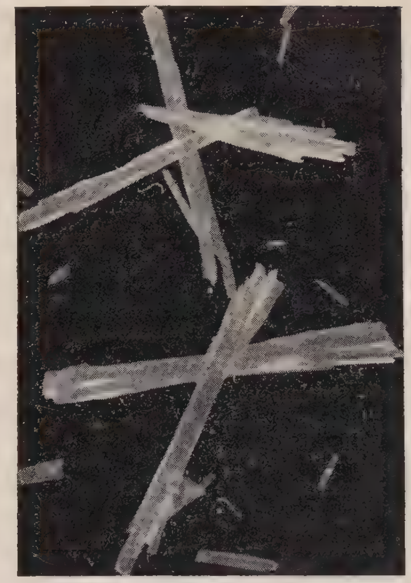

Fig. 4. Oxydiertes Lysozym (lytische Aktivität etwa 60%). Abgedruckt vom Farbenfilm, Vergrösserung ×500.

Fig. 5. Oxydiertes Lysozym (ohne Aktivität), Vergrösserung ×200.

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[13] H.L. Fevold, "Advances in Protein Chemistry," Vol. VI, S. 187, Academic Press Inc., Publishers, New York, N.Y. (1951).

pH 5.4 aufgelöst, dann centrifugiert und noch dazu filtriert. Zu 30 ccm von dem Filtrate, dessen Eiweisskonzentration 0.12% war, nach Abkühlen bis 4°C, wurde die berechnete Menge von abgekühlter 0.015 N $NaJO_4$ Lösung (0.1 N. Phosphat-Puffer von pH 6.8) zusetzt. Das Gemisch wurde bei 4–5°C liegen gelassen. Nachdem Perjodat, nötigenfalls mittels des Zusetzens von wenigem Traubenzucker, reduziert worden war, wurden zur Lösung Kochsalz, bis seine Konzentration 5 % betrug, dann verd. Natronlauge zugesetzt, um pH von Lösung 9.5–9.8 zu regulieren. Der dabei entstandene Niederschlag wurde centrifugiert und mit der 5%iges Kochsalz enthaltenden verd. Natronlauge von pH 9.5 wiederholt gewaschen, und zuletzt wieder in verd. Essigsäure von pH 5.4 aufgelöst. Aus der dabei erhaltenen Lösung kristallisierte sich oxydiertes Enzym allmählich, indem man ihre Kochsalz-Konzentration zu 5% und pH zu 9.6 regierte.

Im Falle, dass die Belassungstemperatur höher, oder die Reaktionsdauer länger war, war die Kristallisation schwierig. Das übermässig oxydierte Lysozym war gelb gefärbt($\lambda_{max}=400 m\mu$).

### 3. Eigenschaften von oxydiertem Lysozyme, Veränderungen der Zusammensetzung und des UV-Absorptionsspektrums infolge der Oxydation.

a) Wiedererlangen verlorener Aktivität mittels des Cysteins oder des Schwefelwasserstoffs :—Oxydiertes Lysozym, welches die Aktivität von 0 bzw. 75% besass, wurde in dem 1 M. Phosphat-Puffer von pH 6.8 mit Cystein (5mg Cystein/10γoxy.Lysozym), oder Schwefelwasserstoff behandelt (10 Minuten lang durchblasen, dann 5 Minuten lang im Vakuum entfernt wurde). Die Tafel I zeigt die dabei gewonnenen Resultate.

Tafel I

Die verlorene Aktivität wird durch Reduktion nicht wiederhergestellt.

| | Lytische | Aktivität |
|---|---|---|
| Behandlung mit $H_2S$ | 0 | 70 % |
| Ohne $H_2S$ | 0 | 75 % |
| Cystein-Zusatz | 0 | 75 % |
| Ohne Cystein | 0 | 75 % |

Tafel II

Veränderung in Zusammensetzung infolge der Oxydation

| | Lysozym14)15) | Oxydiertes Lysozym |
|---|---|---|
| Cystin | 6.8 % | 6.8 % |
| Histidin | 1.04 % | 1.0 % |
| Tryptophan | 10.6 % | 0 7 % |

Die verlorene Aktivität des mit Perjodat oxydierten Lysozyms konnte man durch Reduktion nicht wiederher-

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14) J.C. Lewis, N.S. Snell, D.J. Hirschmann und H. Fraenkel-Conrat, *J. Biol. Chem.*, **186**, 23 (1950).

15) C. Fromageot, M. Privat de Garilhe, *Biochim. et biophys. Acta*, **4**, 509 (1950).

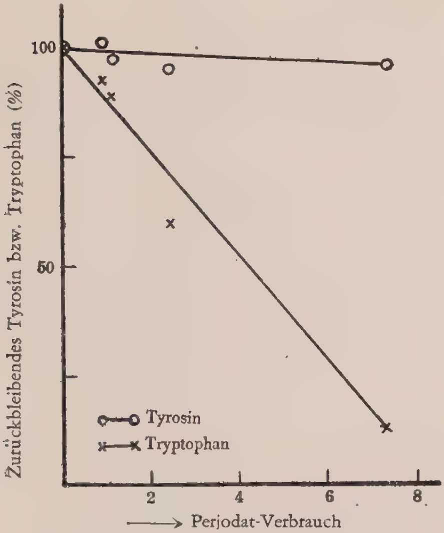

Fig. 6. Die Zersetzung des Tryptophans bzw. Tyrosins infolge der Oxydation.

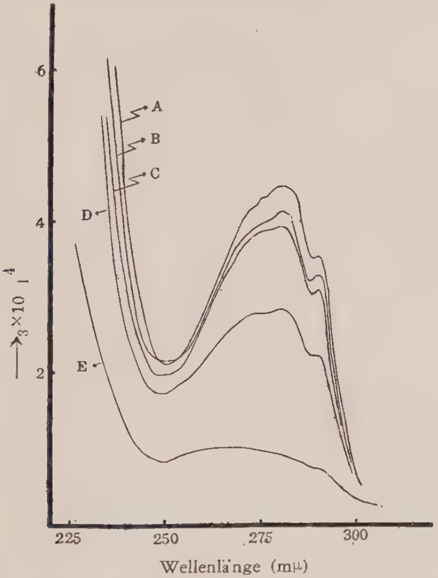

Fig. 7. Absorptionsspektren von Lysozyme und von dem in verschiedenem Grade oxydierten Lysozyme. Die Aufnahme der Spektren wurde in der 0.1 N. Phosphat-Puffer Lösung von pH 6.4 durchgeführt.
A···Lysozym. B···Oxydiertes Lysozym (0.905 Mol $NaJO_4$ verbr./Mol Lysozym). C···Oxydiertes Lysozym (1.01Mol $NaJO_4$ verbr./Mol Lysozym). D···Oxydiertes Lysozym (2.42 Mol $NaJO_4$ verbr./Mol Lysozym). E···Oxydiertes Lysozym (7.30Mol $NaJO_4$ verbr./Mol Lysozym).

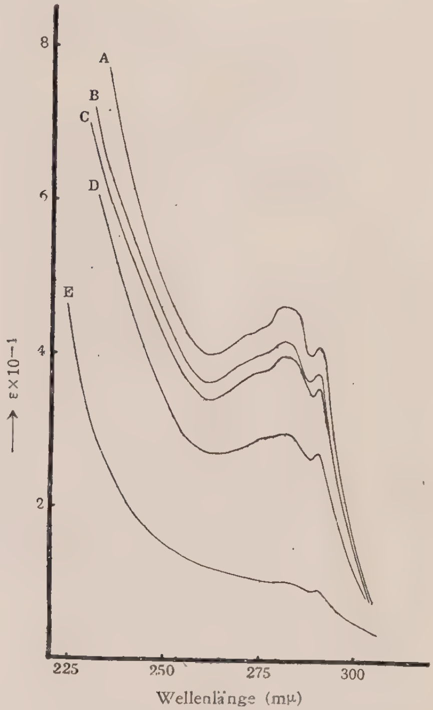

Fig. 8. Absorptionsspektren von Lysozyme und von dem in verschiedenem Grade oxydierten Lysozyme. Die Aufnahme der Spektren wurde in der Lösung von 0.1N. Natronlauge durchgeführt.
A···Lysozym. B···Oxydiertes Lysozym (0.905 Mol $NaJO_4$ verbr./Mol Lysozym). C···Oxydiertes Lysozym (1.01 Mol $NaJO_4$ verbr./Mol Lysozym). D···Oxydiertes Lysozym (2.42 Mol $NaJO_4$ verbr./Mol Lysozym). E···Oxydiertes Lysozym (7.30Mol $NaJO_4$ verbr./Mol Lysozym).

stellen, nicht nur in der schon seine Aktivität verlorenen Probe, sondern auch in der noch aktiven.

b) An den Aktivität gerade verlorenen Oxyproben wurden Tyrosin[18], Tryptophan[18], Cystin[16] und Histidin[17] bestimmt. Die Resultate sind in der Tafel II zusammengestellt. In der Figur 6 wurde weites Resultat gezeigt.

Man kann erkennen, dass das Try-

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16) E.S.G. Barron, "Adv. in Enzymol.", Vol. 11, S. 223, Interscience Publishers, Inc., New York, N. Y. (1951).

17) A. Hunter, *J. Biol. Chem.*, **196**, 589 (1952).

18) T.W. Goodwin und R.A. Morton, *Biochem. J.*, **40**, 628 (1946).

ptophan in dieser Probe zwar angegriffen wird, aber Cystin und Histidin fast unverändert bleiben.

c) Nachdem man durch wiederholte Umkristallisation oder Fällung das in verschiedenem Grade oxydierte Lysozym jedes für sich von Jod-Verbindung befreit hatte, wurde es in 0.1 N. Phosphat-Puffer von pH 6.4 bzw. in 0.1 N. Natronlauge aufgelöst. Die Absorptionsspektren wurden mit dem Beckman'schen Quarzspektrophotometer aufgenommen (Fig. 7 und 8).

Der Vergleich der Absorptionsspektren der verschiedenen oxydierten Proben zeigt, dass ein Gipfel bei 280.5 m$\mu$ von der Grundform losgerissen worden ist. Die Absinkung des Gipfels bei 280.5 m$\mu$ ist mit der Abnahme der biologischen Aktivität verbunden (vgl. Fig. 1).

## *Zusammenfassung*

Unter milden Bedingungen wurde das Lysozym durch Perjodat oxydiert und oxydiertes Lysozym kristallisierte sich.

Es ist zu folgern, dass das Tryptophan sich auf früher Stufe im Verlaufe der Oxydation zersetzen lässt.

Die Abnahme der Aktivitat und das Zersetzen des Tryptophans infolge der Oxydation stehen in einer geradlinigen Beziehung.

Dieser Versuch wurde mit Unterstützung von Hrn. M. Hasegawa, und mit Hilfe der Ausgeben des Unterrichtsministeriums für Wissenschaftliche Forschung durchgeführt.

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*Ehime Universität*

[Eingegangen am 19, Okt. 1954]

# 6. *Studies on Nucleic Acid and Related Substances*

## *Part 1, The Determination of Nucleic Acids in Yeasts and the Other Microorganisms*

By Yoshiro KUROIWA and Yu HORIE

Many methods for the estimation of nucleic acids have been reported. From among them, Ogur and Rosen's method has been selected because of its excellent precision and ease of treatment. Their method has been confirmed as being applicable for the determination of nucleic acids of dried yeasts, moulds and bacteria, but it has given erroneous results when applied directly to fresh or durable yeasts. It has been proven that the extraction of RNA from fresh or durable yeasts was not carried out completely unless the samples were subjected to a preliminary treatment with dry ice and ether. Analytical data obtained by the present method of determining RNA and DNA in various microorganisms has been presented.

### *Introduction*

Many studies have been reported on the method for determination of nucleic acids in tissues. Various difficulties, however, have been encountered in employing them for the determination in microorganisms. In nearly all the methods available except the cytochemical one, nucleic acid content has been calculated from the determined values of phosphorus, sugar or purine. It is well known that these components have been found not only in nucleic acids, but also in various compounds of living cells. It is, accordingly, necessary to study whether or not hitherto reported methods will give as accurate amounts of nucleic acids in yeasts and the other microorganisms as in the animal tissues.

A scrutiny of the literature on the determination of nucleic acids in microorganisms reveals that the following criticism must be taken into account. Methods based on phosphorus determination may give rise to certain erroneous results, since Schmidt and Thanhauser[1] noted that "the determinations of nucleic acid by phosphorus should be supplemented by ribose or purine determination." And Wiame studied metaphosphate[2,3,4,5] and reported that metaphosphate[6,7] of microorganisms has a solubility very similar to that of nucleic acids. Values obtained by methods based on sugar determination were reported by Thomson et al.[8] to be higher compared to those determined by ultraviolet spectrophotometric method. His results may be caused by polysaccharide[9] present in cells. In the case of microorganisms, the polysaccharide must be taken into consideration. It has generally been recognized that the estimation of nucleic acids by purine determination[10,11,12,13] requires "hours of delicate work" as Jeener and Brachet[10] or Di Carlo and Schultz[14] commented and lacks precision.

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[1] G. Schmidt, L. Hecht and S.J. Thanhauser, *J. Biol. Chem.*, **166**, 775 (1946).

[2] J.M. Wiame, *Bull.soc.chim.biol.*, **28**, 552(1946).

[3] J.M. Wiame, *Federation proc.*, **6**, 302 (1947).

[4] J.M. Wiame, *J.Amer.Chem.Soc.*, **69**,3146(1947).

[5] J.M. Wiame, *Biochim. et Biophys. Acta*, **1**, 234 (1947).

[6] M.G. McFahlene, *Biochem. J.*, **40**, 1369 (1946).

[7] T. Mann, *Biochem. J.*, **38**, 345 (1944).

[8] R.Y. Thomson, F.C. Heagy, W.C. Hutchison and J.D. Davidson, *Biochem. J.*, **53**, 460 (1953).

In order to determine nucleic acids in yeasts and the other microorganisms, chemical methods based on phosphorus or sugar determination have usually been employed[15,16,17,18,19,20] after a sample has been homogenized with Pyrex glass powder in sodium chloride solution. But Sato[21] modified Schmidt and Thanhauser's method to remove errors due to polysaccharide and Di Carlo and Schultz[14,22,23] introduced a spectrophotometric procedure considering the above mentioned defects. The latter method, however, can not be widely used for microorganisms, because it was studied only on yeasts and so their DNA (desoxyribonucleic acid) content was neglected. On the other hand, Ogur and Rosen's[24] method presented for plant tissues seemed to be appropriate as to precision and feasibleness of the treatment. But they recognized that their method was not suitable for *Saccharomyces*,, applying their method to *Saccharomyces*.[25] They did modify their method indicating that "a portion of DNA was extracted by the cold perchloric acid treatment yielding a cross-contamination of the RNA (ribonucleic acid) fraction."

In the present investigation a study has been made to modify Ogur and Rosen's method[24] so as to be suitable for determinations in fresh microorganisms. It has been found that the extraction of RNA from fresh or durable yeasts was not accomplished unless the samples were subjected to a preliminary treatment with dry ice and ether. Data obtained by the present method of determining RNA and DNA in yeasts and the other microorganisms has been presented.

### *Method for Estimation of Nucleic Acids in Yeasts and other Microorganisms*

In the case of dried yeasts, moulds and bacteria, Ogur and Rosen's method[24] can be used without any modification. In fresh or durable yeasts, the following modified method should be employed.

The aliquot, equivalent to 50-250mg of solid matter, is weighed in a centrifugal tube, its water content adjusted to 80 per cent by adding water, 2 g of dry ice and 2 cc of ethyl ether are added, and frozen at −70°C overnight and kept in Dewar vessel filled with dry ice. Then, "alcohol-soluble", "alcohol-ether-soluble" and "acid-soluble compounds" are removed from the sample in the centrifugal tube according to the procedure described in Ogur and Rosen's method.[24)]

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[9] E. Hammersten, *Acta Med. Scand.*, Suppl. (No. 196) **128**, 634 (1947).

[10] R. Jeener and J. Brachet, *Enzymologia*, **11**, 222 (1944).

[11] R. Vendrely and R. Sarciron, *Bull. soc. chim. biol.*, **26**, 214 (1944).

[12] H.M. Kalckar, *J. Biol. Chem.*, **167**, 429 (1947).

[13] P. Decker and K. Dirr, *Biochem. Z.*, **316**, 249 (1944).

[14] F.J. Di Carlo and A.S. Schultz, *Arch. Biochem.*, **17**, 294 (1948).

[15] A. Bourdet, P. Mandel and R. Guillemet, *Compt. rend.*, **232**, 756 (1951).

[16] G. Leonardi, *Compt. rend.*, **229**, 393 (1949).

[17] H.B. Levy, E.T. Skutch and A.L. Shade, *Arch. Biochem.*, **24**, 206 (1949).

[18] H.v. Euler and L. Hahn, *Arkiv. Kemi. Mineral. Geol.*, **24A** (28), 1 (1947).

[19] R. Jeener, *Biochim. et Biophys. Acta*, **8**, 125 (1952).

[20] E. Chargaff, S. Zamenhof, G. Brawerman and L. Kerin, *J. Amer. Chem. Soc.*, **72**, 3825 (1950).

[21] H. Sato, *Tohoku J. Exptl. Medr*, **55**, 53 (1951).

[22] F.J. Di Carlo, A.S. Schultz and R.A. Fischer, *Archiv. Biochem.*, **20**, 90 (1949).

[23] F.J. Di Carlo, A.S. Schultz and R.A. Fischer, *ibid.*, **27**, 29 (1950).

[24] M. Ogur and G. Rosen, *ibid.*, **25**, 262 (1950).

[25] M. Ogur, S. Minckler, G. Lindegren and C.C. Lindegren, *Archiv. Biochem. Biophys.*, **40**, 175 (1952).

In order to extract RNA, the residue thus obtained is suspended in 5 cc of 1 N perchloric acid and stored at 4°C with occasional stirring. After fifteen hours the residue is repeatedly extracted with 5cc of the acid every one-two hours. The extraction is continued for about twenty-four hours, and all the centrifugates and washings are made up to 50 cc. To extract DNA the final residue is suspended in 5 cc of 0.5 N perchloric acid and warmed at 70°C in a water bath for twenty minutes. This hot extraction is repeated, thereby washing the residue with the hot acid. All the centrifugates and washings are combined and made up to 25 cc. The RNA and DNA fractions are diluted with 1N and 0.5 N perchloric acid respectively to make their phosphorus level range from 0.5 to 2.0 $\mu$g P per cc. The optical density is measured at 260m$\mu$ and 268 m$\mu$ by Beckman spectrophotometer model DU using 10mm absorption cells. If the optical densities measured be $x$ and $y$, respectively, the phosphorus content of RNA and DNA of the sample can be calculated by the following formulae:

$$\mathrm{RNAP} = a \cdot x \cdot 1/0.343\ \mu\mathrm{g},$$

$$\mathrm{DNAP} = b \cdot y \cdot 1/0.293\ \mu\mathrm{g},$$

where, $a$ and $b$ indicate each dilution factor, RNAP, ribonucleic acid phosphorus and DNAP, desoxyribonucleic acid phosphorus.

*Experimental*

### 1. Ultraviolet Extinction of RNA and DNA.

Prior to examining the analytical procedure, it is necessary to have the correct ultraviolet extinctions of RNA and DNA. Several commercial samples and the authors' preparations were dissolved in aqueous solutions at a level of 20mg per cent. The phosphorus contents of these solutions were determined by Allen's[26)] method. These sample solutiens were diluted with 1 N and 0.5 N

Table I

Ultraviolet Extinction of Nucleic Acid Samples

RNA: in 1N perchloric acid. DNA: in 0.5N perchloric acid after being heated for 20 mins. at 70°C. Absorption cell: 10mm.

| RNA |  |  | DNA |  |
|---|---|---|---|---|
| Sample |  | E*(1 $\mu$g P/cc) | Sample | E(1 $\mu$g P/cc) |
| Author's prepn. | a | 0.348 | Minophagen | 0.296 |
|  | b | 0.342 | Nutritional Biochemicals | 0.290 |
| E. Merck |  | 0.341 |  |  |
| Fleischman |  | 0.347 |  |  |
| Evans |  | 0.339 |  |  |
| Mean value |  | 0.343 |  | 0.293 |
| Ogur and Rosen's value |  | 0.350 |  | 0.283 |

* Optical density

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26) R.J.L. Allen, *Biochem. J.*, **34**, 858 (1940).

perchloric acid respectively to a level of 1 $\mu$g of nucleic acid phosphorus per cc and the optical densities were determined by using Beckman spectrophotometer at 260 m$\mu$ for RNA and 268 m$\mu$ for DNA based on Ogur and Rosen's data. Recently, Logan[27] made a detailed study of the behavior of the maximum absorption for RNA and DNA under various conditions. This is remained beside the question this time.

Data given in Table I shows a slight difference in values for extinction of RNA and DNA from those given by Ogur and Rosen, but the present mean values were used throughout the work.

### 2. The Extraction of RNA and DNA from Microorganisms by Ogur and Rosen's Method.[24]

It has often been noticed that even water-soluble constituents of microorganisms, especially of fresh yeasts, could not be completely extracted by plasmolysis.[28,29,30] Such a phenomenon is expected particularly in cases where the RNA has a higher molecular weight. An attempt has been made to extract nucleic acids from fresh yeasts, dried yeast and other microorganisms, by preliminary treatment with the glass

Table II

Comparison of Nucleic Acid Contents in Microorganisms Obtained by Ogur and Rosen's Method with or without Pretreatment.

| | Sample | Untreated | Homogenized* | Freezing method proposed |
|---|---|---|---|---|
| i) | Fresh yeast | | | |
| | RNAP | 0.589 | 0.578 | 0.611 |
| | DNAP | 0.351 | 0.0400 | 0.0180 |
| | DNAP by DA** | 0.018 | 0.018 | 0.018 |
| ii) | Dried yeast | | | |
| | RNAP | 0.439 | 0.421 | 0.437 |
| | DNAP | 0.0471 | 0.0505 | 0.0475 |
| | DNAP by DA | 0.047 | 0.047 | 0.047 |
| iii) | *Penicillium chrysogenum* | | | |
| | RNAP | 0.232 | 0.102 | 0.233 |
| | DNAP | 0.0196 | 0.0342 | 0.0194 |
| | DNAP by DA | 0.019 | 0.019 | 0.019 |
| iv) | *Aspergillus oryzae* | | | |
| | RNAP | 0.222 | 0.199 | 0.222 |
| | DNAP | 0.0268 | 0.0362 | 0.0267 |
| | DNAP by DA | 0.027 | 0.027 | 0.027 |
| v) | *Balillus mesentericus* | | | |
| | RNAP | 0.289 | 0.209 | 0.295 |
| | DNAP | 0.110 | 0.114 | 0.108 |
| | DNAP by DA | 0.108 | 0.108 | 0.108 |

* treated by glass homogenizer using Pyrex glass powder.
** data obtained by diphenylamine reaction.
All the figures for phosphorus are expressed as per cent on dry basis through Table II -VI.

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27) J.E. Logan, W.A. Mannel and R.J. Rossiter, *Biochem. J.*, **51**, 470 (1952).

28) Y. Kuroiwa, "Extraction of Thiamine from Yeast", unpublished data.

29) Y. Kuroiwa, U. Ito, H. Horiguchi and S. Shimamoto, *J. Japan Soc. Food Nutrition*, **2**, 23 (1949).

30) Y. Kuroiwa, *ibid.*, **2**, 13 (1949).

31) V.R. Potter and C.A. Elvehjem. *J. Biol. Chem.*, **114**, 495 (1936).

homogenizer[31]. As will be seen from data presented in Table II, the extraction of RNA from fresh yeast by Ogur and Rosen's method was incomplete and the remaining RNA was carried over to the DNA fraction by hot extraction, as already indicated by Patterson[32], thus introducing an appreciable error even if estimated by spectrophotometric method. This is evidence by remarkably higher values for DNA compared with those obtained by diphenylamine reaction.[33] Table II also shows that a complete extraction of RNA by Ogur and Rosen's method took place from dried yeast and the other microorganisms. This is also evidenced by the fact that values for DNA obtained by Ogur and Rosen's method are in conformity with those obtained by the present freezing procedure as well as by the diphenylamine reaction. It is interesting to note that in contrast to RNA of dried yeast that of fresh or durable yeast could not be extracted easily. This incomplete extraction of RNA may be due either to a state of the cell membrane or to some physiological characteristics of fresh or durable yeast cells which are quite different from those of dried yeast cells.

The preliminary homogenizing of microorganisms with Pyrex glass powder has been widely used for the extraction of nucleic acids[17,20]. It has been found, however, in the present study that this homogenizing caused incomplete extraction of RNA to take place, resulting in a higher value for DNA. Since it has been confirmed that no adsorption of RNA on Pyrex glass powder occurred, no satisfactory explanation for this unexpected phenomenon can be made so far.

### 3. Appropriate Condition for Extraction by the Freezing method.

Kuroiwa[30] devised the "dry ice method" to carry out efficient isolation of glutathione from yeasts since the ordinary plasmolytic treatment did not give a promising result. In order to apply this method to the extraction of nucleic acids from fresh or durable yeasts, factors governing degree of the extraction have been studied. As shown in Table III, it was found necessary to keep the yeasts frozen at least overnight. Attempts have also been made to destroy the cell membrane by alternate repetition of freezing with dry ice and melting.

Table IV shows that incomplete extraction resulted even after fourty repetitions of freezing and melting.

Furthermore, an autolytic pretreatment has been tried with unsatisfactory results as will be seen from Tabte V.

It remained to be solved that values for RNA or DNA obtained after the freezing treatment represent the respec-

Table III

Effect of Duration of Frozen State on Extraction of Nucleic Acids.

| | Untreated | Frozen for | | | |
|---|---|---|---|---|---|
| | | 3 hrs. | 5 hrs. | 18 hrs. | 42 hrs. |
| RNAP | 0.589 | 0.598 | 0.605 | 0.611 | 0.611 |
| DNAP | 0.0351 | 0.0252 | 0.0211 | 0.0180 | 0.0183 |
| DNAP by DA* | 0.018 | 0.018 | 0.018 | 0.018 | 0.018 |

* data obtained by diphenylamine reaction.

Table IV

Effect of Alternate Repetitions of Freezing and Melting of Fresh Yeast

| | Freezing method proposed | Freezing-melting method (40 times repetition) |
|---|---|---|
| RNAP | 0.608 | 0.549 |
| DNAP | 0.0235 | 0.0656 |

Table V

The Effect of 3 hr.-Autolysis at 40°C under Toluol on Extraction of Nucleic Acid from Fresh Yeast.

| | Uutreated | Freezing method proposed | Autolysis |
|---|---|---|---|
| RNAP | 0.551 | 0.554 | 0.421 |
| DNAP | 0.0363 | 0.0288 | 0.0418 |
| DNAP by DA | 0.029 | 0.029 | — |

Table VI

Estimation of RNA and DNA in the RNA and DNA Fractions by Diphenylamin and Orcine[34] Reactions.

| | Sugar test | | Freezing method proposed |
|---|---|---|---|
| | RNA | DNA | |
| RNA-fraction | 0.645 | 0.000 | 0.647 |
| DNA-fraction | — | 0.020 | 0.0207 |

tive true values for RNA and DNA. Ogur and Rosen demonstrated that when cells are destroyed, RNA could be completely extracted with cold 1 N perchloric acid and DNA with warm 0.5 N perchloric acid. Data presented in Tables II, III, V and VI, clearly show that values for DNA obtained after being subjected to the freezing procedure can be considered as being true values from the fact that they were in good conformity with those estimated by the diphenylamine reaction. And the estimation by orcine and diphenylamine reactions of DNA and RNA in the RNA fraction obtained by the freezing procedure reveals that no DNA exists in the RNA fraction. It will be concluded, accordingly, that the present freezing pretreatment is adequate for the complete extraction of RNA and DNA.

### 4. The Nucleic Acid Contents of Yeasts and Some Other Micro-organisms.

By employing the present freezing method, the nucleic acid contents of several microorganisms have been estimated. The samples used were all in the resting state. As shown in Table VII, values for RNA/DNA are higher in yeasts, lower in moulds, and lowest in bacteria. These results are consistent with those of previous workers.[35] In the case of fresh yeasts, values for RNA/DNA showed a wide

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32) E.K. Patterson and M.E. Dackerman, *Archiv. Biochem. Biophys.*, **36**, 97 (1952).

33) Z. Dische, *Mikrochemie*, **8**, 4 (1930).

34) S.E. Kerr and K. Seraidarian, *J. Biol. Chem.*, **159**, 211 (1945).

35) R. Vendrely and Y. Lehoult, *Compt. rend.*, **222**, 1357 (1946).

Table VII

Nucleic Acid Contents of Various Microorganisms.

| | Sample | RNA* | DNA | RNA/DNA |
|---|---|---|---|---|
| a) | Brewers' yeast | 6.44 | 0.182 | 35.3 |
| | ,, | 5.20 | 0.312 | 16.6 |
| | ,, | 6.82 | 0.210 | 32.4 |
| | ,, | 4.62 | 0.207 | 22.3 |
| | ,, | 5.43 | 0.245 | 22.1 |
| b) | Bakers' yeast (Fleischman) | 4.16 | 0.220 | 18.9 |
| c) | *Saccharomyces Sake* | 3.62 | 0.442 | 8.19 |
| d) | *Saccharomyces anamensis* | 2.67 | 0.238 | 11.2 |
| e) | Dried yeast | 4.78 | 0.378 | 12.8 |
| | ,, | 4.61 | 0.516 | 8.93 |
| f) | *Aspergillus oryzae* (shaking culture) | 2.34 | 0.270 | 8.66 |
| | ,, (surface culture) | 0.665 | 0.300 | 2.21 |
| g) | *Aspergillus niger* | 0.982 | 0.607 | 1.61 |
| h) | *Rhizopus nigricans* | 1.85 | 0.322 | 5.74 |
| i) | *Penicillium chrysogenum* | 2.46 | 0.196 | 12.5 |
| j) | *Absidia glauca* | 1.94 | 1·32 | 1.47 |
| k) | *Bacillus subtilis* | 0.297 | 0.731 | 0.406 |
| l) | *Bacillus mesentericus* | 3.11 | 1.10 | 2.82 |
| m) | *Escherichia coli* | 1.35 | 4.50 | 0.300 |

* RNAP was converted to RNA and DNAP to DNA by dividing by 0.095 and 0.099[36] respectively, for ease of comparison with data reported by previous workers.

variation and were not always in good agreement with those by Bourdet et al.[15] In *Aspergillus oryzae* a wide variation in RNA and DNA contents and values for RNA/DNA was also observed depending on the culture conditions. These variations even within the same species may be caused by the difference in physiological states of microorganisms.

### *Summary*

1. It has been confirmed that Ogur and Rosen's method can be applied to the determination of nucleic acids of dried yeasts, moulds and bacteria.
2. It has been found that their method gives erroneous results when applied directly to fresh or durable yeasts.
3. Correct results have been obtained when fresh or durable yeasts were subjected to freezing overnight with dry ice and ether.
4. In order to effect the complete extraction of nucleic acid, such preliminary treatments as autolysis, homogenizing with Pyrex glass powder, and alternate repetitions of dry ice -freezing and melting have been tried with no promising results.
5. Based on the experimental results, a most reliable procedure for the determination of nucleic acids of microorganisms has been described.
6. Analytical data of RNA and DNA in various microorganisms obtained by the present method has been presented.

### *Acknowledgement*

The authors would like to thank Messrs Yoshio Otsuka and Masuo Tokikuni, the managing directors of

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[36] W.C.Schneider, *J. Biol. Chem.*, **161**,293(1945).

Kirin Brewery Company, who gave us the opportunity to publish this report. We are grateful for many suggestions and the continued interest of Mr. Takeo Kumazawa, the president of Kirin Research Institute and Dr. Yuzuru Okuda, a member of the Japan Academy and the former president of this institute.

*Kirin Research Iustitute,*
*Amagasaki Laboratory, Amagasaki*

[Received, Oct. 21, 1954]

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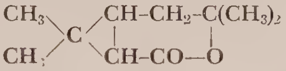
# 7. *Studies on Chrysanthemic Acid.*

## *Part 1. Syntheses of Some Related Substances of Chrysanthemic Acid*

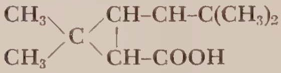
By Masanao MATSUI and Seiichi KITAMURA

For the purpose of synthesizing "dihydrochrysanthemolactone", homocaronic anhydride was reacted with methylmagnesium iodide. The product was not the lactone, but it's isomer, *dl-cis*-2,2-dimethyl-3-(1-methyl-1-hydroxyethyl)-cyclopropaneacetic acid lactone.

2,2-Dimethyl-3-(2-methyl-2-hydroxypropyl)-cyclopropanecarboxylic acid lactone or "dihydrochrysanthemolactone" (I) was first obtained by S.H. Harper[1] by dilute sulfuric acid treatment of *cis*-chrysanthemic acid (II).



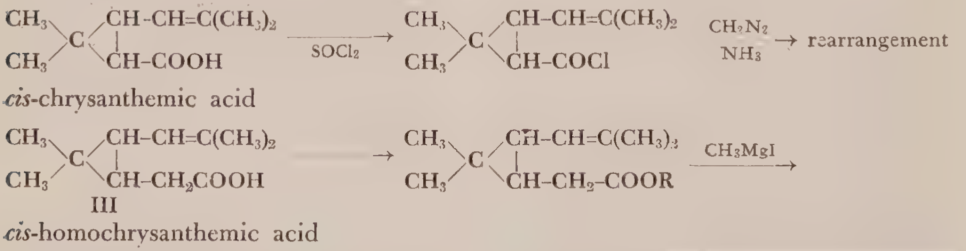
I



II

Our attempts to synthesize the lactone through another route, different from that of S.H. Harper, has been in vain, but enough experimental data has been collected for the publication of this report containing some new information.

Our first attempt to synthesize the lactone was by the following scheme.



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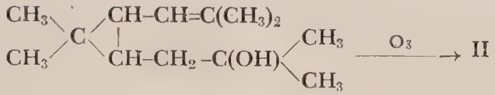
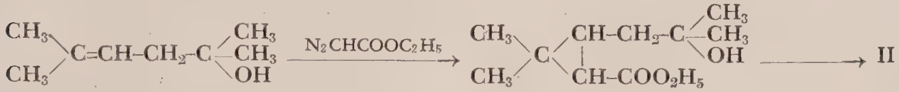
[1] S.H. Harper, *Pyrethrum Post*, January and April (1945), **1**, No. 3, 9–15.
L. Combie, S.H. Harper and R.A. Thompson, *J. Sci. Food. Agric.*, **1951**, No. 9, 421.
S.H. Harper and R.A. Thompson, *ibid.*, **1952** No. 5, 230.

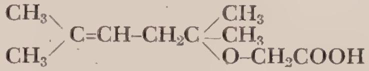


We obtained III as a crystalline acid melting at 78–79°C, but the yield was poor. As a result we were prevented from performing the next process.

The second method was as follows:

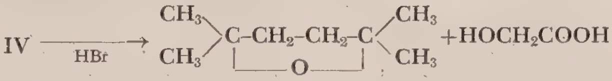


But this reaction also failed. The resulting acid was 2,5-dimethyl-4-hexenyl-2-oxy-acetic acid IV.

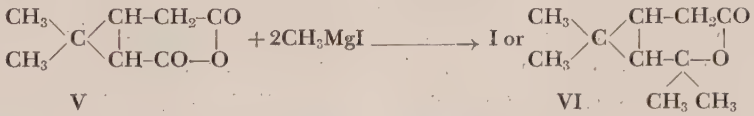


IV

The structure was proven by hydrolysis of the acid with hydrobromic acid. The products were 2,2,5,5-tetramethyltetrahydrofuran and glycolic acid which was characterized by crystalline *p*-bromophenacyl ester, m.p. 139°.



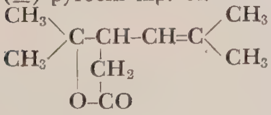
The third method was one starting from *cis*-homocaronic anhydride (V).



V

VI

We obtained two lactones in this preparation from homocaronic anhydride and methylmagnesium iodide. One melts at 41° and the other at 48°. Anaylsis showed the two lactones have the same empirical formula $C_{10}H_{16}O_2$. If two carbonyls of homocaronic anhydride were about equally reactive, one

| | $KMnO_4$ in acetone | $Br_2$ in acetic acid | 15% $Na_2CO_3$ solution |
|---|---|---|---|
| lactone mp. 41° | does not decolorize | does not decolorize | insoluble |
| lactone mp. 48° | decolorizes after a few minutes | slowly decolorizes | insoluble |
| (±) dihydrochrysanthemolactone mp. 62°1) | does not decolorize | does not decolorize | insoluble |
| (±) pyrocin mp. 62°2)  | decolorizes | decolorizes at once | insoluble |
| (±) *cis*-chrysanthemic acid mp. 112° | decolorizes | decolorizes at once | highly soluble |

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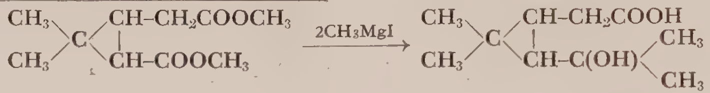
2) This compound was derived from chrysanthemic acid by heat treatment, Masanao Matsui, Takashi Ohno, Seiichi Kitamura, Misao Toyao; *Bul. Chem. Soc. Japan*, **25**, 210 (1952).

of the two lactones had to be identical with dihydrochrysanthemolactone and the other had to be the substance VI. But each of them was different from the dihydrochrysanthemolactone.

The chemical behavior of the two lactones and of some compounds related to chrysanthemic acid are listed as follows :

The comparison of the above data shows that the lactones melting at 41° is almost undoubtedly VI, and the lactones melting at 48° seems to have a saturated linkage. If the latter has a double bond instead of a cyclopropane ring, it might be due to the crude oily homocaronic anhydride which contaminated some unsaturated substances derived from homocaronic acid by heat treatment. However, we cannot conclude that the lactone melting at 48° has no cyclopropane ring; further investigation are necessary to confirm the structure of the lactone.

We also synthesized *trans*-2,2-dimethyl-3-(1-methyl-1-hydroxyethyl)-cyclopropaneacetic acid (VIII) melting at 63° starting from methyl *trans*homocaronate (VII) by a method similar to that used in the synthesis of *cis*-lactone (VI).



VII VIII

## *Experimental*

### 1. *cis*-Homochrysanthemic acid

Fifteen grams of *cis*-chrysanthemoyl chloride were dropped at 0° with stirring into the dry ether solution of diazomethane prepared from 35 g. of nitrosomethylurea, 350ml. of ether and 90ml. of 50 % potassium hydroxide solution. After one hour, excess diazomethane was decomposed by dropping a small quantity of acetic acid into the solution. One hundred ml. of 25 % ammonia, 300ml. of alcohol and 6g. of silver nitrate in a small quantity of water were added and ether was distilled off (350ml.). Twenty ml. of 25 % ammonia were added again and the mixture was warmed at 75–80° on a water bath. After the evolution of nitrogen ceased, 10 ml. of saturated sodium chloride solution were added. After filtration, 20 g. of potassiumhydroxide were added and the mixture was refluxed for 15 hours. Then 100ml. of water was added and alcohol was distilled off. The mixture was extracted with ether, and neutral substances were discarded. The water solution was acidified with dilute sulfuric acid and extracted with ether. The ether extract was washed with water and dried over anhydrous sodium sulphate. After evaporation of ether, the residue was distilled and the fraction distilling at 145–156°/14 mm. was collected, yield 9 g. *Analysis :* found ; C, 72.39; H, 9.99 % calcd. for $C_{11}H_{18}O_2$ C, 72.35; H, 9.8%. A part of the above distillate crystallized, m.p. 78–79°, *Analysis :* found ; C, 72.43; H, 10.01 ; calcd. for C, 72.53 ; H, 9.89 %. *p*-Bromophenacyl ester was prepared but the melting point was not sharp and recrystallization was difficult, mp. 47–55°.

### 2. Reaction between 2,5-dimethyl-2-hexen-5-ol and ethyl diazoacetate

Thirty five grams of 2,5-dimethyl-2-hexen-5-ol[3] and 1 g. of copper powder were heated at 150–160° in an oil bath and 35 g. of ethyl diazoacetate was added dropwise. After addition heating was continued until the evolution of nitrogen ceased. The reaction mixture was fractionally distilled, the following fractions being collected: *a*) 70–100°/15 mm. $n_D^{19}$ 1.4387, 6.5 g. *b*) 100–130°/15mm., $n_D^{19}$ 1.4439, 13g. *c*) 130–157°/15mm., $n_D^{19}$ 1.4482, 13 g. *d*) 150–156°/7 mm., $n_D^{19}$ 1.4527, 10 g. *b*) *c*) and *d*) were combined (32 g.) and saponified with 30g. of potassium hydroxide and 150 ml. of ethanol by refluxing for 3 hrs. Water was added and alcohol was distilled off, neutral substances were extracted with ether and discarded. The water solution was acidified with dilute sulfuric acid and the acid was extracted with ether. The ether extract was washed with water and dried over anhydrous sodium sulfate. After evaporating the solvent, the residue was distilled under reduced pressure. *e*) 113–135°/5mm., $n_D^{16}$ 1.4603, 6g. *f*) 128–142°/5 mm., $n_D^{16}$ 1.4615, 2.5g. *g*) 142°/5mm., $n_D^{16}$ 1.4652, 3g. *Analysis* of f); found C, 64.99; H, 9.68; calcd. for $C_{10}H_{18}O_3$; C, 64.52; H, 9.68%. The same *p*-bromophenacyl ester (mp. 54–55°) was obtained from fractions e) and f) *Analysis*: found; C, 57.03; H, 6.42; calcd. for $C_{18}H_{23}O_4Br$; C, 56.24; H, 6.25%.

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[3] Judefind and Reid, *J.Am.Chem.Soc.*, **42**, 1055, (1920).

Two grams of fraction e) was heated at 140° for 2 hrs. with 10 ml. of 40% hydrobromic acid in a sealed tube. The reaction mixture was extracted with ether and the ether extract was distilled. About 0.5g. of oil which distilled at 113° was obtained. It had properties of 2,2,5,5-tetramethyltetrahydrofuran. *Analysis:* found C, 75.45; H, 12.60; calcd. for $C_8H_{16}O$ C, 75.00 H, 12.50%.

The water layer of the ether extraction was evaporated to dryness in a dish on a water bath. Some water was added and the solution was neutralized with 250 mg. of potassium hydroxide in 1 ml. of water, then 900mg. of *p*-bromophenacylbromide and 10 ml. of alcohol was added and the mixture was boiled on a water bath for one hour. Crystals were separated and recrystallized from 94% alcohol mp. 139°. *Analysis:* found C, 44.07; H, 3.16; Br, 30.13; calcd. for $C_{10}H_9O_4Br$ C, 44.0; H, 3.3; Br, 29.3%. This was *p*-bromophenacyl ester of glycolic acid[3].

### 3. *dl-cis*-2,2-Dimethyl-3-(1-methyl-1-hydroxyethyl)-cyclopropaneacetic acid lactone (VI) and *dl-trans*-2,2-Dimethyl-3-(1-methyl-1-hydroxyethyl)-cyclopropaneacetic acid (VIII).

#### 1) *dl-cis-* and *trans*-Homocaronic acids.[4]

Ethyl pyroterebinate was prepared by the method of J. Owen and J.L. Simonsen[5], bp. 80°/30 mm. The ester was condensed with ethyl diazoacetate using copper powder as the catalyst[5].

One hundred nine grams of the ester and 2 g. of copper powder were placed in a 500 ml. three necked flask to which a condenser, a thermometer and a dropping funnel were attached. When the temperature of the contents reached 120° the heat source was removed and dropwise addition of ethyl diazoacetate was started. J. Owen and J.L. Simonsen carried out this reaction at 50°. But the reaction proceeded very slowly at 50°. The higher reaction temperature (120°) was more time saving than the method of J. Owen and J.L. Simonsen.. The dropping rate of ethyl diazoacetate was controlled so that the temperature did not rise above 125° without external heating. When the evolution of nitrogen ceased, the contents were decanted into a Claisen flask and distilled, and the distillate up to 110°/30mm. was recovered (80g.) and the residue was kept separately. aside. The distillate was treated again with 50g. of ethyl diazoacetate in the same flask used before and the contents were distilled and a distillate up to 110°/30mm, was recovered (65g.), the residue being kept separately. The recovered ester was treated with 45g. ethyl deazoacetate and this time a distillater was treated with 45g. of ethyl deazoacetate and at this time a distillat up to 110°/30mm. was recovered (45g.) and the residue was treated as before: that is to say it was treated with 25g. of diazoester and the distillate (35g.), the greater part of which distilled at 98–105°/10mm., was collected. Isocaprolactone seemed to be generated from ethyl pyroterebinate in the course of the reaction. The residues of the above four runs were combined and distilled and the fraction which distilled at 115°–140°/21mm. was collected, yield 154g.

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[4] J. Owen and J.L. Simonsen, *J. chem. Soc.*, **1933**, 1225.

[5] I.C.M. Cambell and Stanley H. Harper, *J. chem. Soc.*, **1945**, 283.

One hundred fifty four grams of the above ester were boiled on a water bath together with 100g. of potassium hydroxide and 500 ml. of ethanol for 1.5 hrs. Insoluble potassium cyclobutanetatracarboxylate was filtered off and washed with some alcohol. Water was added to the filtrate and all the alcohol was distilled off. After cooling the water solution was acidified with dilute sulphuric acid. After one day crystals were filtered and washed with a small volume of ice-water. The crystals were dissolved in ethyl acetate and insoluble matter was removed. After evaporation of ethyl acetate, the crystals were collected, yield 80g. They began melting at 120° and melted at 184°. They are a mixture of *dl-cis* and *dl-trans* homocaronic acids. *Analysis:* found C, 55.22, H, 6.74; calcd. for $C_8H_{12}O_4$ C, 55.81, H, 6.97%. The *trans*-acid was obtained from the *cis-trans* mixture according to J. Owen and J.L. Simonsen's. method.[5]

### 2) ***dl-trans*-2,2-Dimethyl-3-(1-methyl-1-hydroxyethyl)-cyclopropaneacetic acid (VIII)**

First, methyl *trans*-homocaronate was prepared. Nine and a half grams of *trans*-homocaronic acid, 25ml. of methanol and 2.5cc. of conc. sulfuric acid were boiled on a water bath for 7 hrs. Ice water was added and the mixture was extracted with ether several times. The ether extract was filtered and the solvents were evaporated on a water bath. The residue was distilled under reduced pressure. The ester distilled at 140°/25mm., $n_D^{17.5}$ 1.4552, yield 9.5g. The ester was also prepared from the acid and diazomethane bp. 130°/17mm., $n_D^{17.5}$ 1.4532.

Nine and a half grams of the above ester were dissolved in 100ml. of dry ether and into it ether solution of methylmagnesium iodide which was prepared from 11g. of methyl iodide, 2g. of magnesium and 50 ml. of dry ether was added dropwise while stirring and cooling in an ice water bath. The mixture was left standing overnight.

It was decomposed with a mixture of sulfuric acid and ice. The ether layer was separated and the water layer was extracted with ether several times. The ether extracts were combined and the ether was evaporated. The residue was saponified with 4 g. of sodium hydroxide and 40 cc. of methanol by refluxing for 3 hrs. Water was added and methanol was distilled off. The residual mixture was extracted with ether and neutral substances were discarded. The aqueous solution was acidified with dilute sulfuric acid and was extracted with ether in an extraction apparatus for 20 hours. After evaporation of the solvent from the ether solution, the residue was distilled under reduced pressure, and a fraction between 140–145°/15 mm. was collected: it weighted ca. 4g. It was dissolved in three volumes of petroleum ether and cooled to $-60°$. It crystallized. It was recrystallized from petroleum ether, mp. 63°, yield 1.5g. *Analysis:* found C, 64.62; H, 9.30, calcd. for $C_{10}H_{18}O_3$; C, 64.51; H, 9.68%. *p*-Bromophenacyl ester mp. 77–79°; *Analysis:* found C, 56.09; H, 6.30, calcd. for $C_{18}H_{23}O_4Br$; C, 56.40; H, 6.10%.

### 3) *dl-cis*-2,2-Dimethyl-3-(1-methyl-1-hydroxyethyl)-cyclopropaneacetic acid lactone (VI)

Five grams of the mixture of *dl-cis* and *dl-trans*-homocaronic acids (mp. 120–184°) were heated with 15cc. of acetic anhydride in a sealed tube at 230° for 3 hrs. The contents were distilled, and homocaronic anhydride distilling at 140°/10 mm. was collected as an oil. A small quantity of the oil was boiled with some water and evaporated to dryness. It crystallized, mp. 130°, recrystallized from water, it melted at 136°. This is *cis*-homocaronic acid. Oily homocaronic anhydride (2.7 g.) was dissolved in 20ml. of dry benzene and while shaking and cooling in an ice-salt bath, the solution was added dropwise to the methylmagnesium iodide prepared from 5.5g. of methyl iodide 1g. of magnesium and 20cc. of dry ether, and the mixture was left standing overnight. It was decomposed with ice and dilute sulfuric acid; then some sodium sulfite was added. The mixture was extracted with ether in an extraction apparatus for 20 hrs. After evaporation of solvents from the ether extract, the residue was distilled and a distillate boiling at 120–140°/12mm. was collected.

A part (500 mg.) of the distillate crystallized in a refrigerator, mp. 41° (from petroleum ether). It is a lactone and dissolves in boiling 30% potassium hydroxide, almost insoluble in cold alkali, insoluble in alkali carbonate. It is *dl-cis*-2,2-dimethyl-2-(1-methyl-1-hydroxyethyl)-cyclopropaneacetic acid lactone. *Analysis:* found; C, 70.94; H, 9.59; calcd. for $C_{10}H_{16}O_2$ C, 71.43; H, 9.69%.

The mother liquor was saponified with 1g. of potassium hydroxide and 100ml. of alcohol by boiling for 2hrs. on a water bath. Some water was added and alcohol was removed by distillation. Neutral substances were extracted with ether in a percolater for 20 hrs. and discarded.

The mother liquor which was removed from the neutral substances by ether extraction was acidified with diluted sulfuric acid, and extracted with ether in an extraction apparatus for 20 hrs. After evaporation of the solvent from the ether extract, the residue was distilled. The main fraction was collected at 155–160°/32mm. (0.8 g.). It was dissolved in 100ml. of ether and washed with saturated sodium bicarbonate solution several times and then with water three times. Ether was removed by evaporation and the residue was dissolved in two volumes of petroleum ether and cooled to −60°. Crystals were collected and recrystallized from petroleum ether, they melted at 47–48° and weighed 0.35 g. *Analysis:* found C, 71.64; H, 9.69. calcd. for $C_{10}H_{16}O_2$; C, 71.43; H, 9.52%. It is lactone but its structure was not confirmed.

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# 8. A Microscopic Study on the Inner Structure of Starch Granules

By I. YAMASAKI, S. UEDA and S. HAYASHIDA

Showing the microphotographs, writers report in this paper that "Black-Koji-amylase system" can easily digest raw starch granules, even that of potato, and by the use of this enzyme system the existence of the lamellation structure in the inner part of potato starch granules can be observed.

It has long been considered that starch granules might ordinarily consist of concentric layers of starch material deposited around a hilum, since striations can be observed under the microscope. Katz[1] based to the hypothesis on the fact that when the heated starch granules were dipped in water, the hardly soluble parts in the granules became swollen and developed the lamellation structure. Frey-Wyssling[2] reported by observing Becke-lines on the potato starch granules that the striations are due to discontinuities in the index of refraction of the deposited material. Within each layer the index of refraction changes continuously downward from inner to outer, but at the border of a layer the index jumps suddenly to a high value. Thus, previous reports on the microscopical structure of the starch granules have dealt almost exclusively with conclusions drawn from the indirect methods mentioned above. In the present report a direct method of observing the inner structure of raw starches is described.

It is well known that native starch granules have a greater resistance to the action of amylase than gelatinized starches, especially raw potato starch which is the most difficult to degrade enzymatically. It has recently been reported from our laboratory[3] that a Black-Koji-amylase system prepared from a certain species of black *Aspergillus*, which is generally used to manufacture Koji in the southern part of Kyushu in Japan, acts much more strongy on raw starch than any other amylase. By means of this enzyme system, it is possible to observe microscopically how various starch granules are degraded. 10cc of M/10 HCl-sodium citrate buffer solution at pH 3.8, 100cc of Black-Koji-amylase solution (a 1:10 extract of Black-Koji in water) and 1cc of toluene were added to 1g of starch granules. After being partly digested for a definite time at 30°C., the starch granules were observed under the microscope. In the above condition, native corn starch granules were wholly dissolved in about 48 hrs., wheat and barley starches in about 125 hrs. Even potato starch granules were completely digested within about two months. In the course of the degradation of the cereal starch granules, as is already well known, many pits appeared on the surface and each pit grew larger and deeper, forming the porous grains. As for potato starch, on the contrary, the intact granules, which really occupy most of the whole, were dissolved away layer by layer, gradually forming long, spindle-shaped grains having clearer striations than the original ones (Fig. 1); but those granules with clefts or fissures were digested preferentially on the damaged spots so as to give rise to channels, pits, cavities, etc. In the latter case, some microphotographs that look into the inner structure of potato starch were obtained.

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[1] N.P.Badenhuizen Jr., J.R.Katz, *Z.Phys.Chem.*, A. **182**, 73 (1933).

[2] A. Frey-Wyssling, "Submikroskopische Morphologie des Protoplasmas und Seiner Derivate." Borntraeger, 278 (1938).

[3] I. Yamasaki, and S. Ueda, *J. Agr. Chem. Soc. of Japan*, **24**, 181 (1951).

Fig. 2 shows that the starch is in layers and that the granule ordinarily consists of these layers. In the cavity on this starch granule, about seven

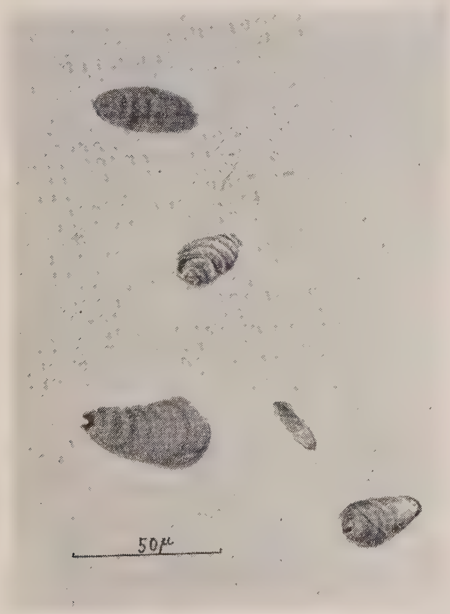

Fig. 1. The intact potato starch granules digested at 30°C. for 3 weeks with Black-Koji-amylase system buffered at pH 3.8

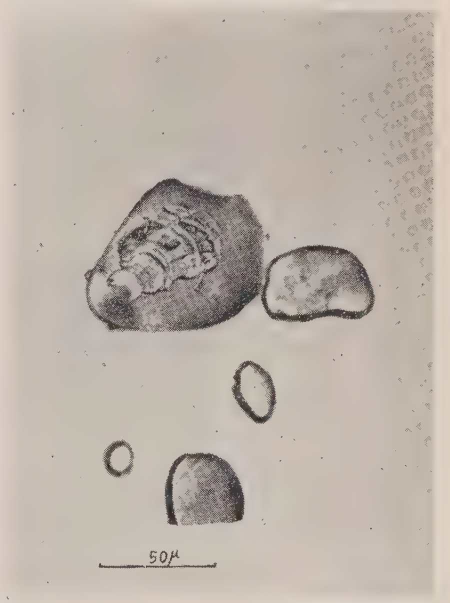

Fig. 2. The damaged potato starch granule digested at 30°C. for 2 weeks with Black-Koji-amylase system buffered at pH 3.8

layers are conspicuous and on each layer many stripes are seen running along radial axes. These may have some relation to the radial molecular arrangement which, it has been concluded from birefringence studies, exists i.e., from the optical anisotropy of the starch granules.

The striations become clearer as the enzymic action proceeds, since the parts located between the striations dissolved away more quickly than the striated parts. If such a tendency progresses to an extreme, the granules may become as shown in Fig. 3. The front side of the granule is lost and seven layers are vividly revealed around the hilum. It is seen here also that the inter-striation parts are more strongly digested away than the striations themselves. This phenomenon presumably corresponds with the discontinuity in the refraction index or density of the granule.

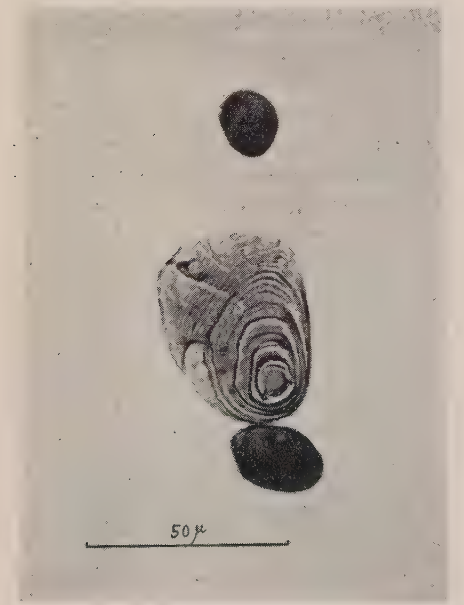

Fig. 3. The damaged potato starch granule digested at 30°C. for 2 weeks with Black-Koji-amylase system buffered at pH 3.8

In Fig. 4, the damaged granule on the left side shows a cross section in which the line at the edge of each layer runs concentrically, and the other down on the right side is probably a fragment cut away along a striation line on the opposite side of the hilum in the granule.

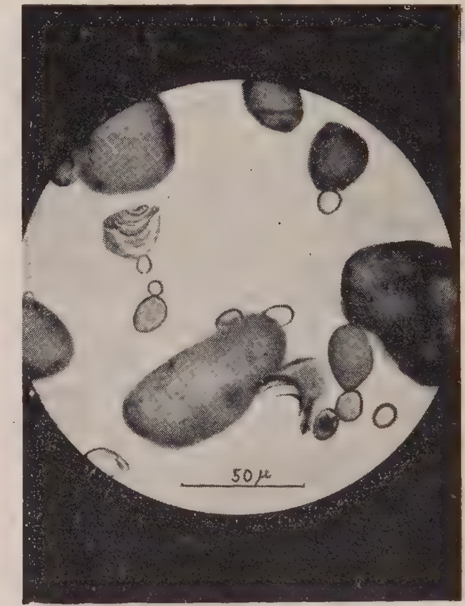

Fig. 4. There are two damaged potato starch granules among intact ones. These are digested at 30°C. for 2 weeks with Black-Koji-amylase system buffered at pH 3.8

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# 9. *Metabolism of Aromatic Compounds by Microbes.*

## *Part 4. Factors influencing adaptive enzyme formation of m-hydroxybenzoic acid and gentisic acid splitting enzymes by* Pseudomonas ovalis var. S-5.

By Kei ARIMA, Kazuo KOMAGATA, Shin-ichi SUGIYAMA,
Masazi KAZAMA and Keiji YANO

For the elucidation of oxidation of *m*-hydroxybenzoic acid and oxidative splitting of gentisic acid by *Pseudomonas ovalis var. S-5,* various factors necessary for the active formation of the adaptive enzymes were examined; i.e. the method of adaptation, the effect of the substrate concentration, the effect of time required for adaptation, and the effect of the energy source in secondary adaptation procedure, and optimum conditions for each of the factors were determined.

### *Introduction*

In the previous report, authors found the two pathway, i.e. the pathways via protocatechuic acid and the pathway via gentisic acid, in the metabolism of *m*-hydroxybenzoic acid by several bacteria, diagnostic studies of which were reported by the present authors.[1,2] The metabolic system involved in the oxidative splitting of protocatechuric acid was first studied by Evans[3] Stanier[4] and the enzyme system responsible for the oxidative splitting of this compound was made manifest by Stanier et al.[5] However, the oxidative splitting of gentisic acid and its metabolic mechanism have not yet been reported. The direct splitting product of gentisic acid is presumed to be fumaryl- or maleylpyruvic acid from several experimental results obtained by the present authors.[6] As the enzymes involved in the oxidation of *m*-hydroxybenzoic acid and gentisic acid are adaptive enzymes, the investigation of the factors which influence active and reproducible formation of the adaptive enzymes were necessary for the basis of the present research i.e., preparation of the cell-free enzyme and its purification, isolation and identification of the direct product of the rupture of gentisic acid from reaction mixture of the pure enzyme, and hence examined in the present report.

### *Experimental Methods*

**Culture :**

Strain. *Pseudomonas ovalis var S-5.*[2]

Medium. Bouillon (pH 7.2) or Bouillon puls 1% glucose

Culture conditions. 30°C, 18 hrs. 100 cc medium in the 500 cc shouldered Sakaguchi flask, reciprocating type shaking culture. 120r.p.m. 7cm.

Harvesting. After centrifugation of the cultured medium, the cells were washed twice with distilled water, and suspended in distilled water and were used as intact cells.

Yield of Cells. Approximately 10g. wet cells were obtained from 100cc bouillon medium culture and 20g. from the same volume of bouillon glucose medium culture.

**Adaptation:**

In order to economize on the expense of the substrate and to use an easy method of analysis of the phenomenon in the induction of enzymes by nonproliferating cells, the secondary adaptation method described as follows was adopted.

**Secondary adaptation method.**

Adaptation medium. M/15 phosphate buffer (pH 7.4) 100cc in the 500cc Sakaguchi flask plugged with cotton.

The substrate. *m*-hydroxybenzoic acid,* M/100 final concentration.

Cells. 2g. as dry weight flask (2mg/cc)

Conditions of incubation. 30°C, 3hrs. unless otherwise stated.

Harvesting the cells. After completion of the induction of adaptive enzymes the cells were separated by centrifugation, washed twice with distilled water, and suspended in distilled water.

The activity of the adaptive cells was determined by Warburg's respirometer from oxygen uptake.

Composition in Warburg's flask.

| | |
|---|---|
| Main compartment. M/15 phosphate buffer (pH 7.4) | 0.5 cc |
| Cell suspension (2.5mg as dry weight/cup) | 0.5 cc |
| Side Arm. M/100 substrate | 0.2 cc |
| Center well. 10% KOH | 0.2 cc |

*In the present study, as the substrate for the induction of the enzymes i.e., *m*-hydroxybenzoic acid oxidase and gentisase, *m*-hydroxybenzoic acid was used exclusively. From the principle of successive or simultanous adaptation, it will be admitted without doubt that *m*-hydroxybenzoic acid has the same value not only for the inducer of *m*-hydroxybenzoic acid oxidase but also for the inducer of gentisase.

From the results obtained, patterns of the enzymatic activity, represented as $O_2$ uptake of the cells on the substrate under various conditions, were drawn, and these patterns will furnish a valuable insight into the adaptive enzyme formation.

The induction of the adaptive enzymes, gentisase and *m*-hydroxybenzoic acid oxidase, were evaluated from the three criterions in the pattern i.e., the length of lag time, $QO_2$ value, and the total $O_2$ uptake. The former two criteria were used exclusively, since the total $O_2$ uptake value does not show directly the activities of the induced enzymes, because it includes the oxidation of substances decomposed further, which is derived from the aliphatic acid which is the direct product of the ring rupture.

## *Experiments and Results*

### Exp. 1. Influence of preadaptation on the enzyme formation.

In order to the economize on the expensive substrates, mainly the secondary adaptation procedures as described were adopted throughout the present experiment. However, the addition of M/200 *m*-hydroxybenzoic acid to the culture medium (bouillon) 3 hours before the harvesting time and addition of M/200 *m*-hydroxybenzoic acid at the start of the culture were carried out and the preadapt cells were processed further in the usual manner in the

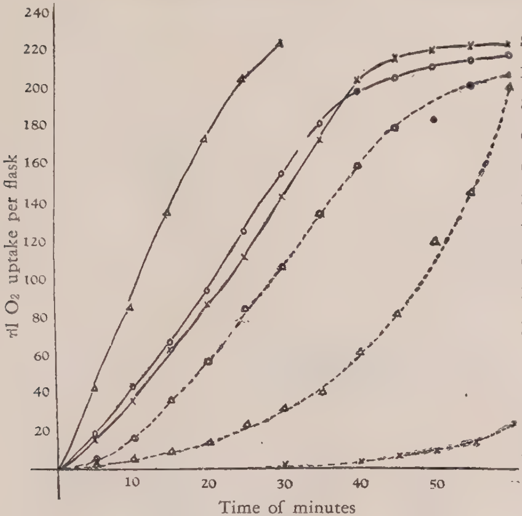

Fig. 1. Effect of the substrate addition to cultural medium 3 hours before harvesting on the induction of *m*-hydroxybenzoic acid oxidase.
Substrate: *m*-hydroxybenzoic acid

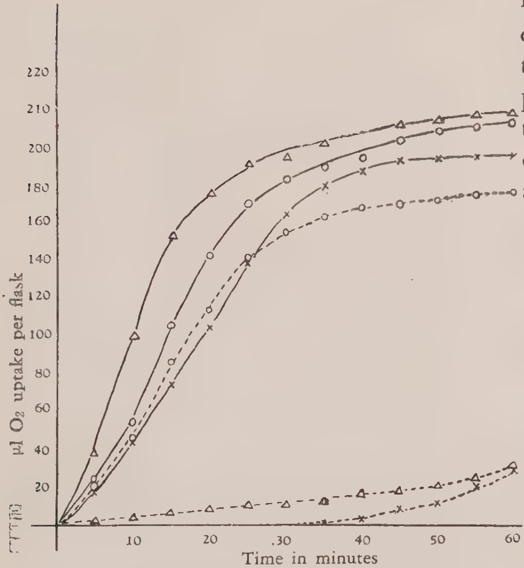

Fig. 2. Effect of the substrate addition to cultural medium at 3 hours before harvest on induction of the adaptive enzyme of gentisic acid oxidation.

Incubation time of the secondary adaptation
Substantial line: The preadapted cell
Dotted line: The secondarily adapted cells as control

| | | |
|---|---|---|
| × —— × | 0 hr. | × ······ × |
| △ —— △ | 1 hr. | △ ······ △ |
| ○ —— ○ | 3 hr. | ○ ······ ○ |

Substrate: gentisic acid.

secondary adaptation procedure. The influence of these two ways of preadaptating to enzyme formation was examined. As the control, the cells from the culture to which no *m*-hydroxybenzoic acid was added were used for the secondary adaptation procedure. The enzyme formation pattern for *m*-hydroxybenzoic acid oxidase and gentisase are shown in Fig. 1 and 2.

As manifested in Figs. 1, and 2, 3 hrs'. preaddition of the substrate remarkably reduced the time required for the induction of *m*-hydroxybenzoic acid oxidase and gentisase in the secondry adaptation process. The zero hour cells in Fig. 1 and 2, that is the cells which were not secondarily adapted, show almost the same activities of the induced two enzymes as those of the control cells which were induced for three hours in the standard adaptation procedure. While in the case of preaddition of the substrate at the start of the culture, one or two hours' secondary adaptation is satisfactory for the com-

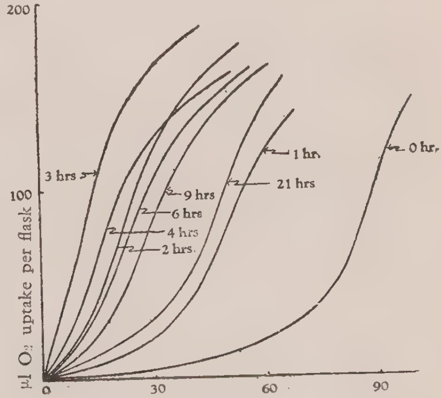

Fig. 3. Influence of time on the adaptibility of *Ps ovalis S-5* for the oxidation of *m*-hydroxybenzoic acid.

Figure in hours illustrates the induction time of the cells in the secondary adaptation procedure.

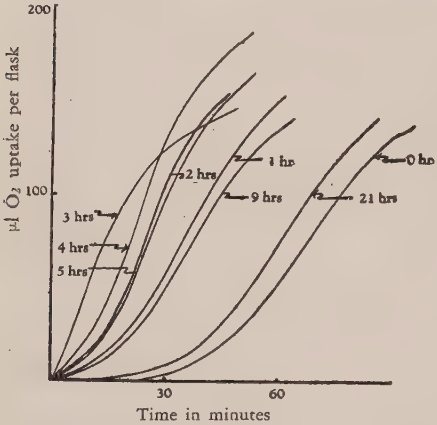

Fig. 4. Influence of time on the adaptability of *Ps. ovalis S-5* for the oxidation of gentisic acid.

Figure in hours illustrates the induction time of the cells in the secondary adaptation procedure.

pletion of the induction of the two enzymes, which in the case of the control necessitates three hours.

### Exp. 2. The influence of time on induction of the enzymes in the secondary adaptation procedure.

Ten grams of wet cells grown on bouillon medium were harvested and washed as described and were processed in the usual manner in the secondary adaptation procedure. During the course of adaptation, the cells were removed from the shaking flask at one hour intervals for 21 hours and the induced enzymatic activities of *m*-hydroxybenzoic acid oxidase and gentisase were determined.

As shown in Figs. 3, 4, 5 the cells at 0 hour i.e. non-adapted cells, have a long lag time, the cells adapted for 1 hour, still show a considerable length of lag time, i.e., 35 min. for *m*-hydroxybenzoic acid oxidation and 15 min. for gentisic acid oxidation. The cells adapted for 2 hours can oxidize both substrates without any lag time. The activities of the two induced enzymes of the cells increased and reached their maximum peak after three hours' adaptation and then again decreased as the adaptive time increased and at about ten hours showed a remarkable length of lag time.

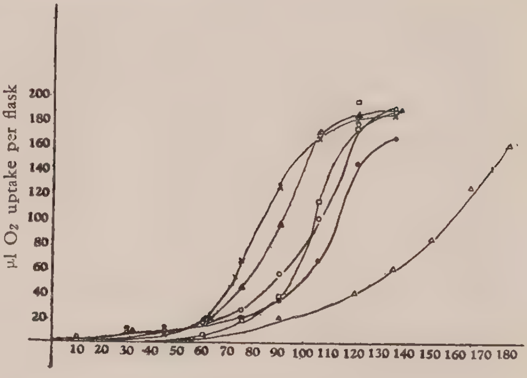

Fig. 5. The adaptive lag time pattern of the induction of *m*-hydroxybenzoic acid oxidase in Warburg's vessel by the cells harvested from the various ages cultured on the glucose bouillon medium.

The age of the culture on bouillon medium when the cells are harvested for the induction of *m*-OH-benzoic acid oxidase in the Warburg's vessel.

○—○— 0 hour △—△— 3 hours
●—●— 7 hours □—□— 15 hours
×—×— 21 hours ▲—▲— 24 hours

In Fig. 5. the influence of time on the adaptability of *Ps. ovalis S-5* using two criterions, i.e., the length of lag time and oxygen uptake values in 10 minute's, were summarized. Under the present experimental conditions the cells adapted for three hours were considered to have the strongest induced enzyme activities.

### Exp. 3. The influence of concentration of the substrate in the secondary adaptation procedure.

The cells grown on bouillon-glucose medium for 18 hrs. were harvested and secondarily adapted for three hours, with the various concentrations of substrate.

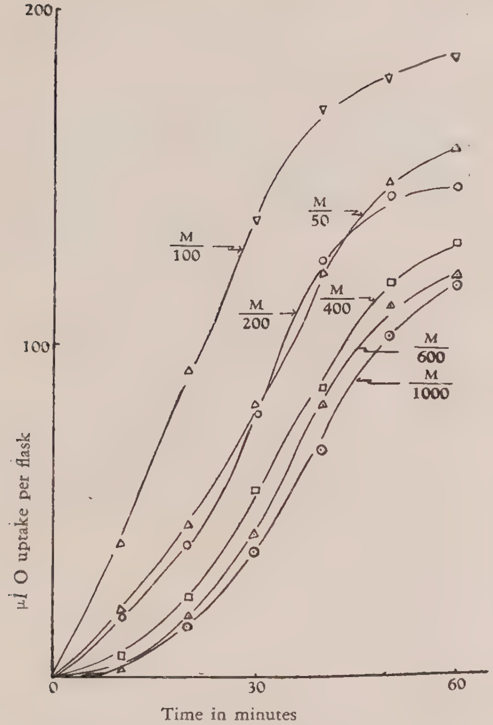

Fig. 6. The effect of substrate concentration on the formation of *m*-hydroxybenzoic acid oxidase.

The substrate in the secondary adaptation procedure: *m*-OH-benzoic acid

The concentrations of the *m*-hydroxybenzoic acid examined in the secondary adaptation procedure were M/50, M/100, M/200, M/400, M/600 and M/1000, and the results obtained are shown in Figs. 6, 7, 8. Judging by the length of lag time and $QO_2$ value, optimum concentration of the substrate for the induction of the enzymes is M/100 for *m*-hydroxybenzoic acid oxidase and M/200 for gentisase.

### Exp. 4. The effect of energy source.

**1) The effect of the glucose concentration at three hours' adaptation.** The cells grown on bouillon for 18 hrs. were used. The glucose concentrations examined were 1/1000 M, 1/750M, 1/500 M, 1/250 M, 1/100 M, 1/50M, and they were added to the flasks in the secondary adaptation procedure respectively. The adaptation time was three hours. The adapted cells were prepared as described, and measured for their induced enzymatic activities by Warburg manometry. The results obtained are shown in Fig. 9 and Fig. 10. Glucose at the concentrations of 1/100M and 1/50M remarkably extended the lag time and decreased the adaptability of the cells. However, at the glucose concentration of 1/1000 M, 1/750 M, 1/500 M, and 1/250M no influence were observed. Therefore, addition of 1/1000M glucose which Suda et al.7) reported as being effective in increasing the angle of inclination of the $O_2$ uptake curve, was not effective either for the induction of gentisase and *m*-hydroxybenzoic acid oxidase under the present experimental conditions.

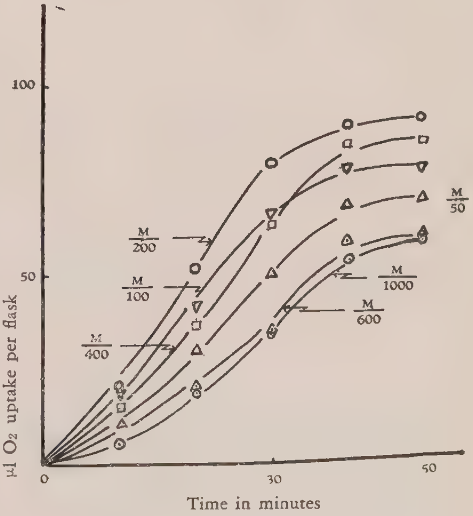

Fig. 7. The effect of substrate concentration on the formation of gentisase.

The substrate in the secondary adaptation procedure: *m*-OH-benzoic acid

**2) The effect of glucose at various adaptation times.** In the former experiment the adaptation time was three hours and concentrations of glucose were changed. In this experiment, the added glucose concentration was in all cases 1/500M, and the cells were adapted for 1, 3, 5, 7 hours and were examined for their ability to induce the adaptive enzymes, i.e., gentisase and *m*-hydroxybenzoic acid oxidase. The cells grown on bouillon for 18 hours were used. As shown in Fig. 11 and 12 at one hour's adaptation the lag time was reduced and the angle of inclination of the $O_2$ uptake curves was increased. Whereas at 3, 5 and 7 hours no remarkable effect was observed upon the induction of the two adaptive enzymes.

**3) The effect of glucose addition on induction of the adaptive enzymes, using starved and non-starved cells in Warburg's vessel.**

In the former experiments the adaptation was carried out in shaking flasks and the cells removed at one or two hours' interval. However this method is not adequate in examining the influence of glucose addition at every period of adaptation, because the interval is long. Therefore a method with which the adaptation and determination of the activity

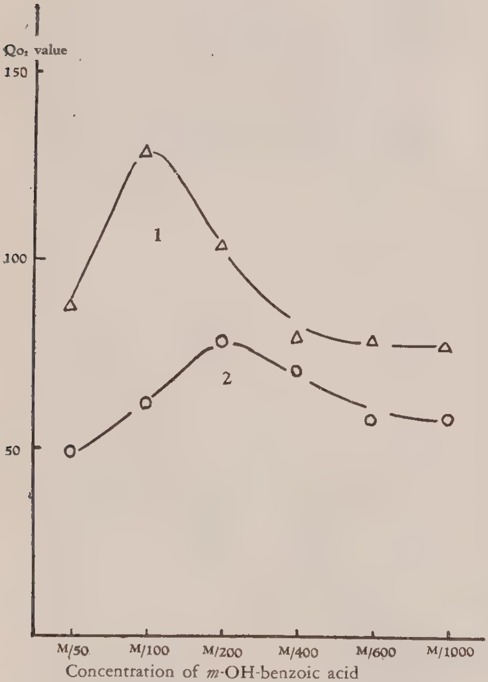

Fig. 8. Influence of concentration of the substrate on adaptability of *Ps. ovalis* for the oxidation of *m*-hydroxybenzoic acid and gentisic acid.

1 *m*-OH-benzoic acid oxidase activity
2 Gentisase activity

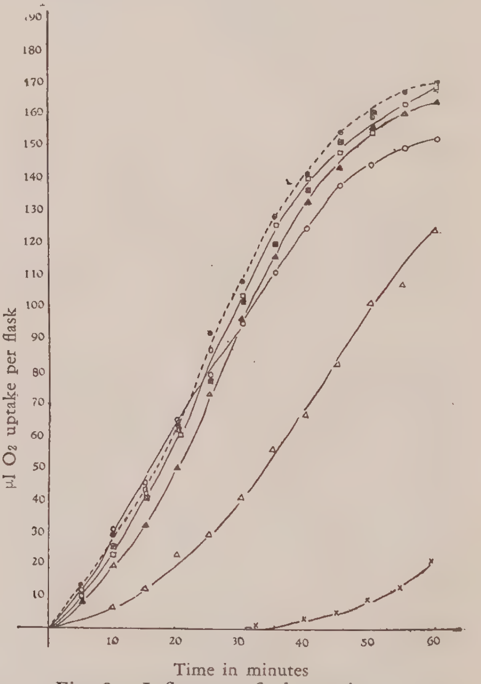

Fig. 9. Influence of the various concentration of glucose on the induction of *m*-hydroxybenzoic acid oxidase in the secondary adaptation in shaken flask.

Concentration of glucose
×—×— M/50 △—△— M/100 □—□— M/250
○—○— M/500 ▲—▲— M/750 ■—■— M/1000
●······●······ control (glucose not added)

of the induced enzymes can be carried out simultaneously in Warburg' vessel was adopted. The cells used were grown in bouillon for 18 hours. The added glucose concentrations examined were M/50, M/1000, M/1500, M/2000.

Composition in Warburg's flask

| | |
|---|---|
| Main compartment | |
| Cell suspension (dry cell weight 2.4mg/cup) | 0.5 cc |
| M/15 phosphate buffer (pH 7.4) | 0.5 cc |
| Centre well | |
| 10% KOH | 0.2 cc |
| Side Arm | |
| 1st. M/100 *m*-OH-benzoate | 0.2 |
| 2nd. glucose solution | 0.2* |

* The final concentration of glucose in the flaks were M/750, M/1000, M/1500, M/2000.

For the controls either M./100 *m*-hydroxybenzoate alone or the glucose solution alone were used.

The method of starvation; the cells from 800ml. bouillon culture broth were resuspended in 100 ml. M/15 phosphate buffer in the Sakaguchi flask

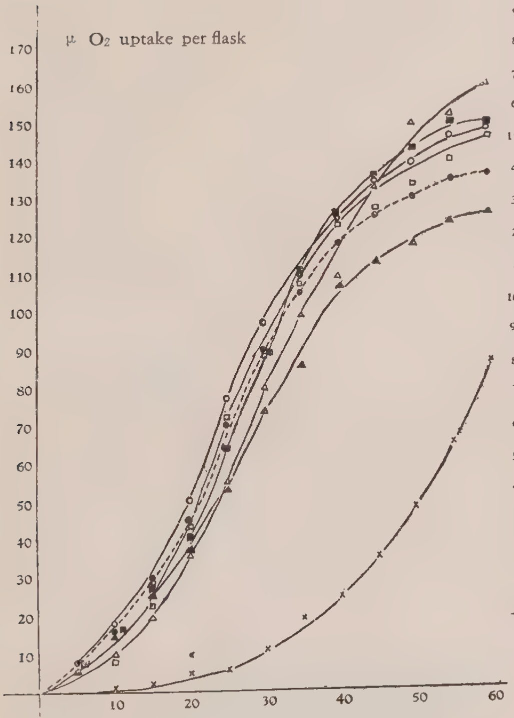

Fig. 10. Influence of the various concentration of glucose on the induction of gentisase in the secondary adaptation in the shaken flasks.

Concentration of glucose.

×—×— M/50　△—△— M/100　□-·-□— M/250

○—○— M/500　▲—▲— M/750　—■—■M/1000

●------●------ Control (glucose not added)

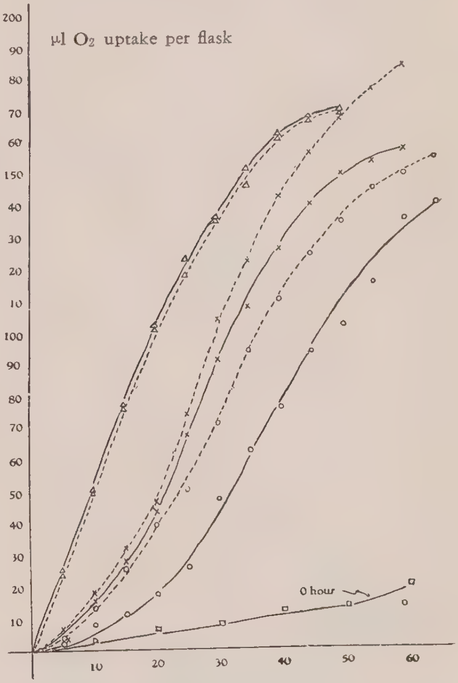

Fig. 11. Influence of the addition of M/500 glucose on the induction of *m*-hydroxybenzoic acid oxidase at various induction time in the secondary adaptation procedure.

Dotted line: M/500 glucose added in the secondary adaptation procedure.

Substantial line: glucose not added.

○—○—, ○------○------ the cells induced for 1 hour.

△—△—, △------△------ the cells induced for 3 hours.

×—×—, ×------×------ the cells induced for 7 hours.

and shaking-cultured for 4.5 hours at 30°C.

The results obtained for unstarved and starved cells are shown in Fig. 13 and 14.

The added glucose in the range of concentrations examined above reduced the lag time but did not increase the $O_2$ uptake rate.

The value of autorespiration of unstarved cells was as high as 70μl. per cup at 100 minutes, whereas the value of the starved cells was still 60μl., therefore the starvation time may not have been long enough.

From the results obtained from these experiments it may be concluded that low concentrations of glucose, such as 1/1000M, was effective on the induction of the two adaptive enzymes in the cells adapted for as short a time as one

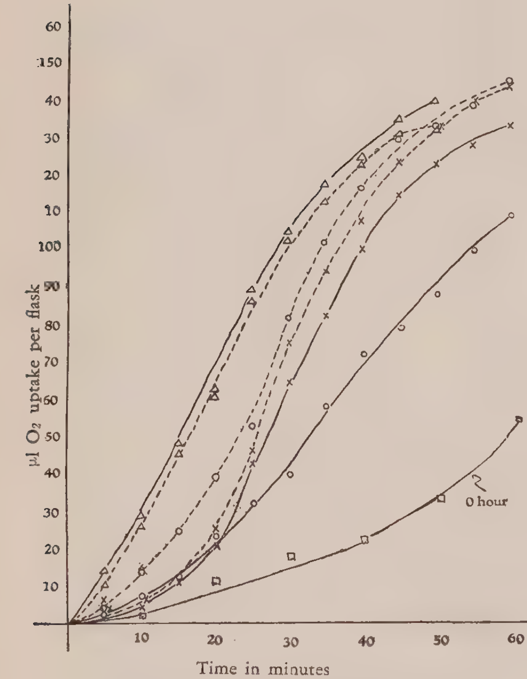

Fig. 12. Influence of the addition of M/500 glucose on the induction of *m*-hydroxybenzoic acid oxidase at various induction time in the secondary adaptation procedure.

Dotted lines: M/500 glucose added in the shaken flasks in the secondary adaptation procedure.

Substantial line: glucose not added.

O—O—, O······O······ the cells induced for 1 hour.
△—△—, △······△······ the cells induced for 3 hours.
×—×—, ×······×······ the cells induced for 7 hours.

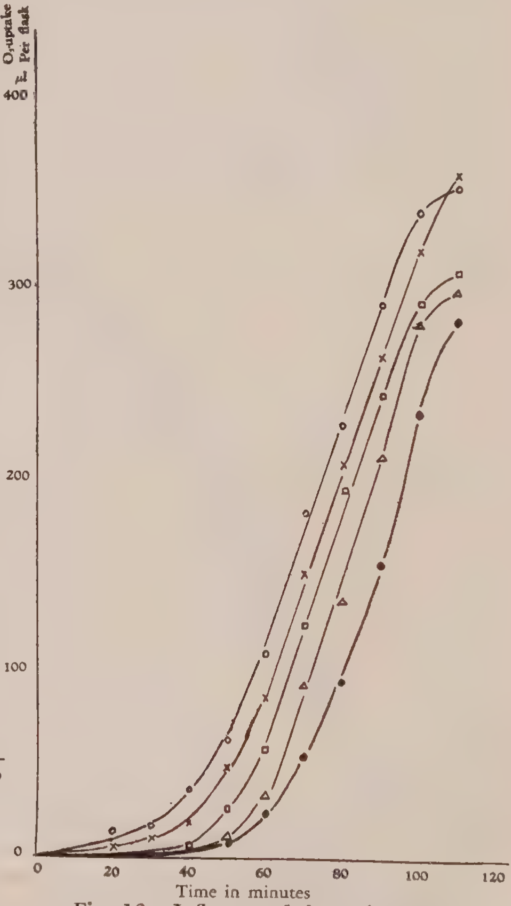

Fig. 13. Influence of the various concentrations of glucose on the induction of *m*-hydroxybenzoic acid oxidase secondarily adaptated in the Warburg's flasks with the cells not starved.

—×—×— M/750 —O—O— M/1000
—□—□— M/1500 —△—△— M/2000
—●—●— Control glucose not added

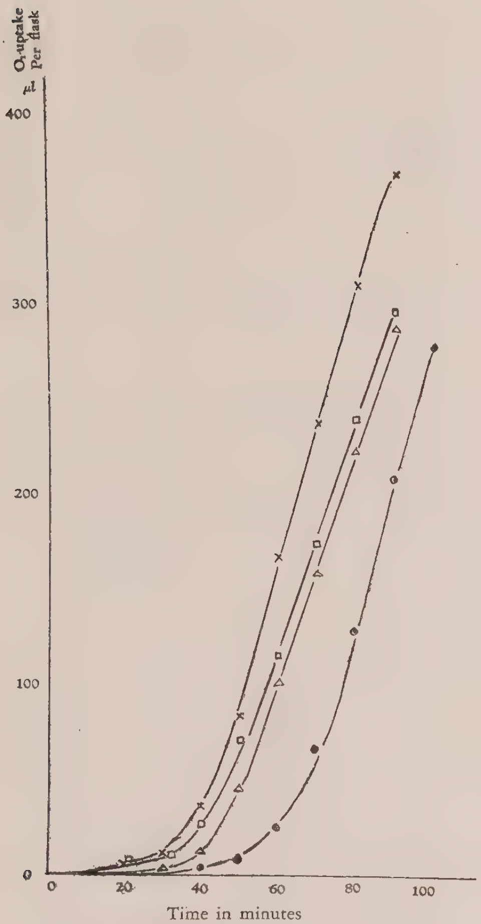

Fig. 14. Influence of the various concentration of glucose on the induction of *m*-hydroxybenzoic acid oxidase secondarily adaptated in the Warburg's flasks with the cells starved for 4.5 hours.

Concentration of glucose
—×—×— M/750 —□—□— M/1500
—|—¦— M/2000
—●—●—Control (glucose not added)

hour in the secondary adaptation procedure and also in the starved and unstarved cells, adapted in Warburg's flasks.

## *Discussion*

The effect of preadaptation was manifest when *m*-hydroxybenzoic acid was added three hours before harvesting, but not when the substrate was added at the start of the culture, presumably because the substrate in the medium was eliminated by the harvesting time. Since the disappearance of substrate causes the rapid breakdown of the once induced adaptive enzymes under the conditions of the shaking culture at 30°C. But even the addition of the substrate three hours before the harvesting is considered to be expensive.

The time required for the induction of adaptive enzymes was longer in the shaking flask in the secondary adaptation procedure than in Warburg's flask, that is 3 hours was optimum for the former method, however the lag time in the Warburg's flask was abous 60 minutes. As the conditions of adaptation are different in each method, it is difficult to compare their discrepancies accurately, but the concentration of the cell suspension in Warburg's flask is about one hundredth of that in the former case. This fact might be a cause for the shorter lag time in the latter method.

The optimum substrate concentration (*m*-hydroxybenzoic acid) was about M/100 in this experiment. However, Suda et al.7) reported that the optimum concentration of benzoic acid for the induction of pyrocatecase in Warburg's vessel was about M/1000.

This discrepancy of the optimum concentration of the substrate may be in part due to the differences in the concentrations of cells in the suspension in which induction of the enzymes were carried out.

The addition of energy sources was reported to be effective by Suda et al.[7]; i.e. the addition of glucose or pyruvate ($2 \times 10^{-4}$ M) remarkably increased the angle of inclination of $O_2$ uptake curve for the oxidation of benzoic acid, though no reduction in the lag time was observed. In his study the effect of the addition of the energy source was more remarkable with cells, whose autorespiration had been decreased, by starvation for one hour at 30°C.

In the present experiment, however, the effect of the energy source was not recognized with cells processed by the standard secondary adaptation method for three hours, through the effect was detectable in one hour's adaptation and adaptation in Warburg's vessels.

The cells starved for four and half hours still show high autorespiration value in *Pseudomonas ovalis S-5*. From the fact that in the cells grown on bouillon-glucose medium, which consequently have a high autorespiration, the addition of energy sources were far from effective but even inhibitive to the induction of the enzymes, so the effect of energy source might not be manifest with the cells of high autorespiration as in this experimental conditions.

### *Summary*

The factors influencing the formation of adaptive enzymes, *m*-hydroxybenzoic acid oxidase and gentisase, in the secondary adaptation procedure by the non-proliferating cells of *Pseudomonas ovalis var. S-5* were examined and the following results obtained.

1) The addition of *m*-hydroxybenzoic acid to the culture media, at the time of initiation of incubation and at three hours before the harvest of the cells, decreases the time required for adaptive enzyme formation in the secondary adaptation, i.e., in the former case 1 to 2 hours and in the latter case 0 to 1 hour is enough for the completion of the induction of both enzymes.

2) The optimum length of time required for the completion of the induction of both enzymes under the standard conditions of the secondary adaptation procedure was approximately three hours.

3) The optimum concentration of substrate, *m*-hydroxybenzoic acid, in the secondary adaptation procedure was M/100 for *m*-hydroxybenzoic acid oxidase and M/200 for gentisase.

4) No effect of the addition of glucose as the energy source upon the induction of gentisase and *m*-hydroxybenzoic acid oxidase was recognized with the cells adapted for three hours in the shaking flask or with the cells grown in bouillon glucose medium. However, the effect was recognized with the cells adapted for one hour in shaking flasks or with the cells adapted in Warburg vessels.

The authors wish to express their sincerest thanks to Prof. K. Sakaguchi, for his guidance and encouragement throughout these research, and also wish to thanks Assist Prof. H. Takahashi for his useful discussion.

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# 10. *Metabolism of Aromatic Compounds by Microbes.*

## *Part 5. Factors influencing adaptive enzyme formation of m-hydroxybenzoic acid and gentisic acid splitting enzymes by* Pseudomonas ovalis var. S-5.

By Kei ARIMA, Kazuo KOMAGATA, Masazi KAZAMA,
Shin-ich SUGIYAMA and Keiji YANO

For the elucidation of oxidation of *m*-hydroxybenzoic acid and oxidative splitting of gentisic acid by *Pseudomonas ovalis var. S-5.* various factors necessary for the adaptive formation of the enzymes, i.e., effect of the age of the culture, effect of oxygen, and effect of inhibitory reagents such as streptomycin, azid, and dinitrophenol against the formation of the adaptive enzymes, were examined. Optimum conditions for each of the above mentioned factors were determined.

### *Introduction*

In the previous paper[1] of this reports, the authors reported various factors which influence the adaptive enzyme formation of *m*-hydroxybenzoic acid oxidase and gentisase in the secondary adaptation procedure which has been used to induce the formation of the adaptive enzymes by nonproliferating intact cells. Optimum conditions were also determined.

In this report, a continuation of the previous reports, the influence of age of the culture, oxygen, and inhibitors upon the formation of adaptive enzymes were determined. It was considered to be of utmost necessity to determine the optimum conditions for the various factors upon the induction of the two enzymes with cell free and purified enzyme preparations.

### *Method*

The strains, the cultural conditions, the method of determination of enzymatic activity of the two induced enzymes, *m*-hydroxybenzoic acid oxidase and gentisase, the secondary adaptation procedure, the adaptation method in Warburg's vessels, and the criteria of adaptability of the cells are all the same as in the previous report.

Glucose : Fehling-Lehmann-Schoorl method

Nitrogen : semi-microkjeldhal method

Turbidity is expressed as the optical density of cells per unit volume of the medium determined electrophotometrically using filter No. 1.

### Exp. 1. The influence of growth phase (age) on enzymatic adaptability of the cells.

#### 1. Growth curve of *Pseudomonas ovaris var. S-5.*

At first, growth curves of the organisms on bouillon and bouillon-glucose medium are examined as in Fig. 1, and

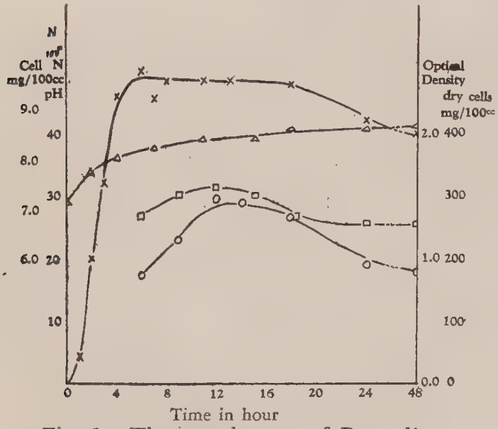

Fig. 1. The growth curve of *Ps. ovalis* var. S-5 grown on bouillon medium by shaking culture.

—×—×— Optical density.
—△—△— pH.
—○—○— Dry cell mg/100 cc broth.
—□—□— Total cellular nitrogen mg/100cc broth.

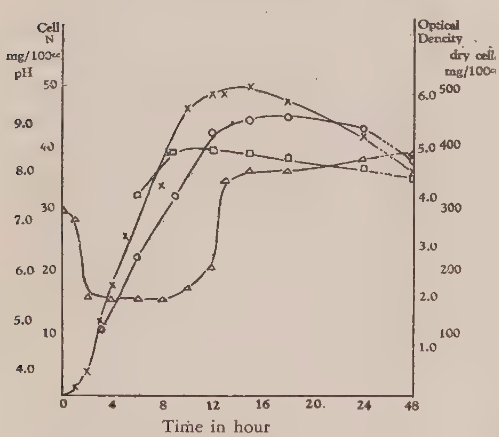

Fig. 2. The growth curve of *Ps. ovalis* var. S-5 grown on glucose bouillon medium by shaking culture.

—×—×— Optical density.
—△—△— pH.
—○—○— Dry cell mg/100 cc broth.
—□--□— Total cellular-Nitrogen mg/100cc broth.

2.

The main differences between the growth curves on bouillon and bouillon-glucose medium are that the latter has a longer log phase, heavier final crops of cells, and low pH during the vigorous sugar consumption stage and a subsequent pH rise.

And furthermore chemical change during culture in the latter medium was more unstable than in the former, for instance the pH curve decreases to the acidic side often will not come up to the alkaline side until the end of the culture.

Cells from such a culture was very weak in enzymatic adaptability.

### 2. The influences of age on enzymatic adaptatibility of the cells grown on bouillon medium.

The cells of two flasks at the culture age of 0, 2, 3, 4, 5, 6, 7, 8, 9, 13, 18 and 24 hrs. were harvested and resting cells from each age were prepared as described. The experiment measuring the adaptability of these resting cells for the induction of *m*-hydroxybenzoic acid oxidase and gentisase were carried out in Wurbug's vessels, according to the method described in the previous report[1]. The results obtained are shown in Fig. 3, 4, 5, 6. The patterns for gentisase show the same tendency and hence omitted here, and only overall relationships of the age of the cells and gentisase induction are shown in Fig. 4. Somewhat contrary to our expectations, the cells from the lag phase, the cells from the beginning of log phase i.e.; so called physiologically young cells, and the cells from the log. phase gave a longer lag time in the induction of the two adaptive enzymes than the cells from the stationary phase. Therefore adaptability of the cells in the stationary phase is considered to be strongest both for *m*-hydroxybenzoic acid oxidase and gentisase. Adaptability of the cells from the end of the stationary phase

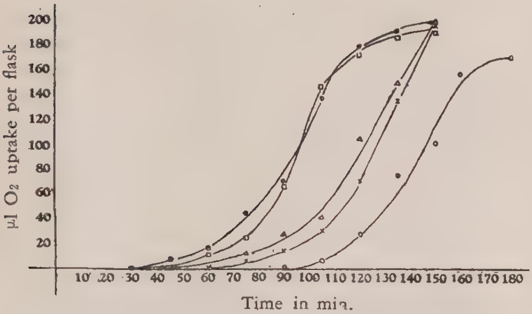

Fig. 3. The adaptive lag time pattern of the induction of *m*-hydroxybenzoic acid oxidase in Warburg's vessel by the cells harvested from the various ages cultured on the bouillon medium.

The age of the culture on bouillon medium when the cells are harvested for the induction of *m*-OH-benzoic acid oxidase in the Warburg's vessel.

| | | | |
|---|---|---|---|
| ○ -○- | 0 hour | △- △- | 4 hours |
| ● ●- | 5 ,, | □-□- | 13 ,, |
| ×-×- | 18 | | |

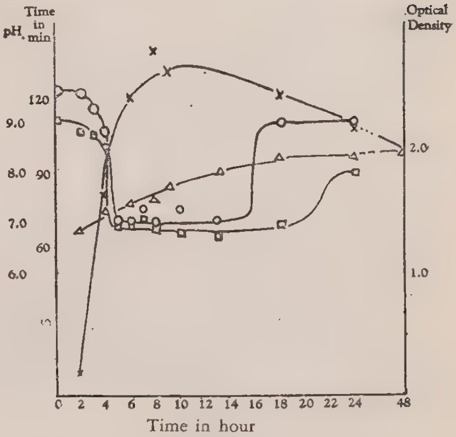

Fig. 4 The relationship between the growth phase and the adaptive lag time for *m*-hydroxybenzoic acid and gentisic acid oxidation. On bouillon medium

- —×—×— Optical density.
- —△—△— pH
- —○—○— lag time for the induction of adaptive enzyme: *m*-OH-benzoic acid.
- —□—□— lag time for the induction of adaptive enzyme: gentisase.

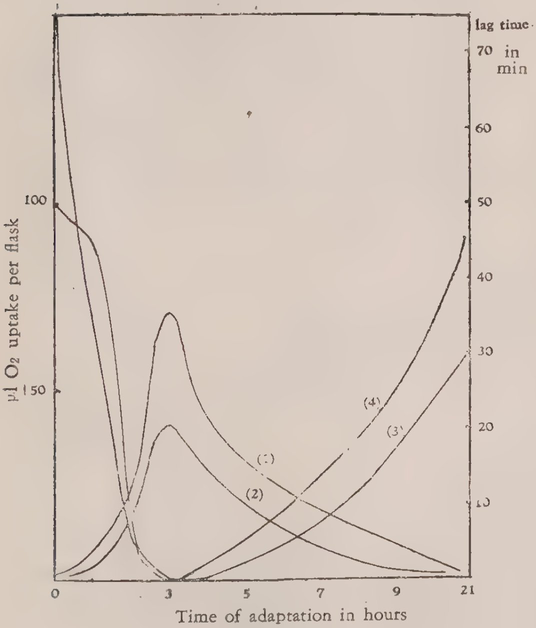

Fig. 5. Influence of time on the adaptability of *Ps. ovalis* as measured by $O_2$ uptake value and length of lag time.

1) $O_2$ uptake value curve in 10 min: Substrate: *m*-OH-benzoic acid
2) $O_2$ uptake value curve in 10 min: Substrate: gentisic acid
3) lag time curve: Substrate: *m*-OH-benzoic acid
4) ,, ,, ,, : Substrate: gentisic acid.

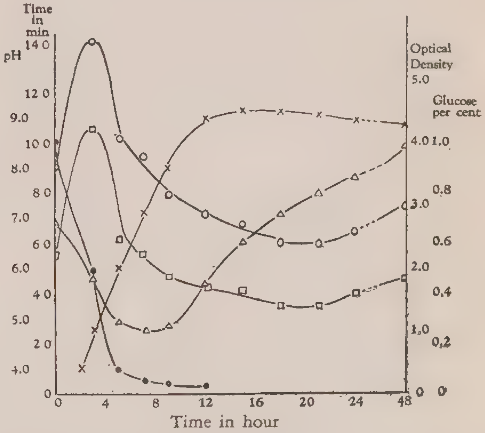

Fig. 6. The relationship between the growth phase and the adaptive lag time for *m*-hydroxybenzoic acid and gentisic acid oxidation. On glucose-bouillon medium.

- —×—×— Optical density.
- —△—△— pH
- —●—●— Concentration of glucose.
- —○—○— lag time for the induction of adaptive enzyme: *m*-OH-benzoic acid oxidase
- —□—□— lag time for the induction of adaptive enzyme, gentisase

again decreased and became very weak; this tendency increases in the cells from the older ages which began to partially autolyse. It was also recognized that the lag times of gentisase were a little shorter than those of *m*-hydroxybenzoic acid oxidase. Overall relationships between adaptability and the age of cells is summarized in Fig. 4.

### 3. The influence of age on the enzymatic adaptability of the cells grown on bouillon-glucose medium.

The same experiment was undertaken with the cells grown on bouillon medium.

Though the growth phase is longer in the bouillon glucose medium than in the bouillon medium, the adaptability tendencies of the cells at each age were almost the same as in the former experiment, as shown in Fig. 5.

The cells from the stationary phase showed stronger adaptability either in $QO_2$ value and length of lag time than the cells from the lag phase, the physiologically young phase and the cells from the log. phase.

The various age groups of the cells can be divided into three groups according to the length of lag time: i.e:

lag time longer than 80 min.
  cells from 3, 5, 7 hrs'. culture
lag time shorter than 70 min.
  cells from 9–15 hrs'. culture
lag time shorter than 60 min.
  cells from 18, 21, 24hrs'. culture

Generally speaking 18, 21, 24 hrs'. culture cells were also high in $QO_2$ value, and hence these cells are considered to have the strongest adaptability. Overall relationships between adaptability and the age of the cells is summarized in Fig. 6.

## Exp. 2. Influence of inhibitors on the formation of adaptive enzymes.

Na-azid, 2,4-dinitrophenol, and streptomycin were added at several points in the course of adaptation and were examined as to effect of inhibition against adaptive formation of the enzymes and also as to the direct inhibition against the enzyme's activity.

### 1. Inhibition by streptomycin.

Before going into the inhibition experiment, the minimum inhibitory concentration of streptomycin upon *Pseudomonas ovalis var. S-5* cultured in bouillon medium (pH 7.0) was examined. The minimum inhibitory concentration of 25 γ/cc at 24 hour culture was found. However, at 48 hours, 50 γ/cc could not inhibit the growth of the organism.

An experiment was carried out in Warburg's vessel by adding a final concentration of $10^{-3}$ M and $10^{-4}$ M streptomycin which approximately corresponds to 25 γ/cc at the starting point of adaptation. The results

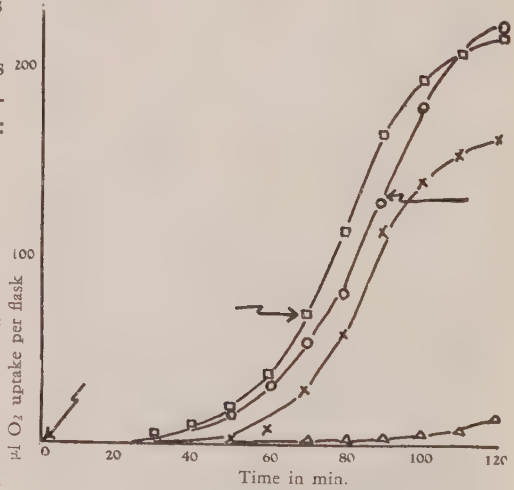

Fig. 7. The influence of streptomycin on the induction of *m*-hydroxybenzoic acid oxidase in Warburg's vessel.
Arrows show the point of streptomycin addition.

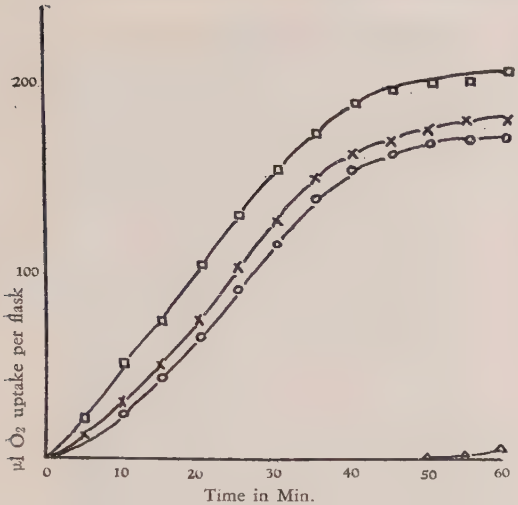

Fig. 8. The influence of the various gases bubbling on adaptive induction of *m*-hydroxybenzoic acid oxidase, Induction time: 3hours.

—□—□— Shaking culture in cotton plugged flasks as the control experiment.
—×—×— Air current bubbled.
—○—○— Oxygen gas bubbled.
—△—△— Nitrogen gas bubbled.

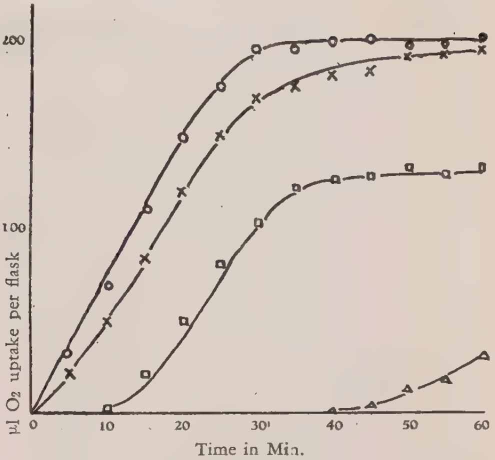

Fig. 9. The influence of the various gases bubbling on adaptive induction of *m*-hydroxybenzoic acid oxidase. Indution time: 1.5 hours.

—□—□— Shaking culture in cotten plugged flask as the control experiment.
—×—×— Air current bubbled.
—○—○— Oxygen gas bubbled.
—△—△— Nitrogen gas bubbled.

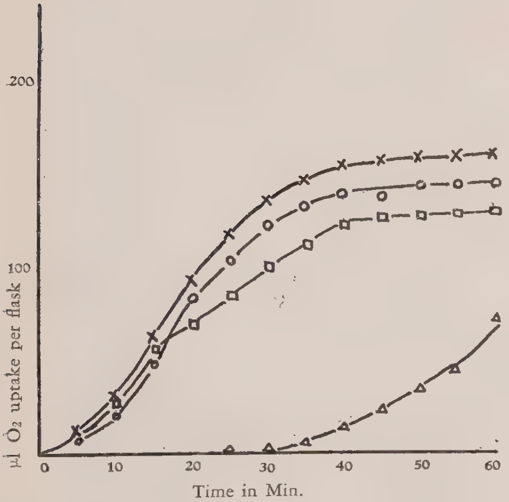

Fig. 10. The influence of the various gases bubbling on adaptive induction of gentisase. Induction time: 3 hours.

—□—□— Shaking culture in cotten plugged flasks as the control experiment.
—×—×— Air current bubbled.
—○—○— Oxygen gas bubbled.
—△—△— Nitrogen gas bubbled.

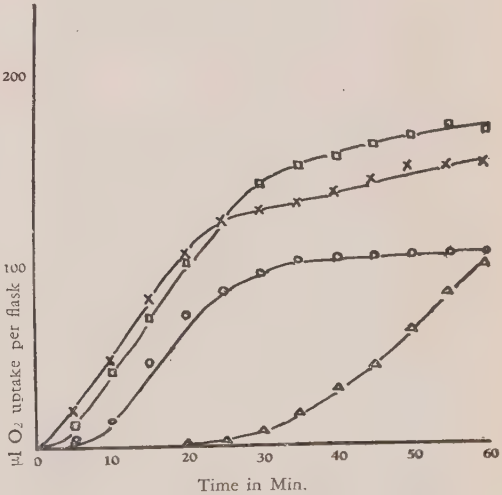

Fig. 11. The influence of the various gases bubbling on adaptive induction of gentisase. Induction time: 1.5 hours.

—□—□— Shaking culture in cotten plugged flasks as the control experiment.
—×—×— Air current bubbled.
—○—○— Oxygen gas bubbled.
—△—△— Nitrogen gas bubbled.

obtained are shown in Fig. 7. The concentration of $10^{-3}$ M and $10^{-4}$ M of streptomycin completely inhibited formation of the adaptive enzymes. Dihydrostreptomycin had the same inhibitory effect as streptomycin at the same concentration. However, once the induction of the two adaptive enzymes has started, the addition of streptomycin i.e., at a relatively early period and later period in the course of induction as shown in Fig. 7, did not inhibit the $O_2$ uptake of this system and hence displays no inhibitory action against the already formed enzymes and also presumably against the formation of the adaptive enzymes.

### 2. Inhibition by 2,4-dinitorphenol and Na-Azide.

The same experiments were undertaken by adding varying concentrations of 2,4-dinitorphenol ($10^{-2}$, $10^{-3}$, $10^{-4}$ M), and Na-azide ($10^{-3}$, $10^{-4}$, $10^{-5}$ M) to the starting, middle, and end points in the course of enzyme adaptation.

However, inhibition occured only when the high concentration of $10^{-2}$M Na-azid was added at the starting point. No inhibition was noticed when any other concentration was added at any point of the adaptation curve. Since $10^{-2}$M is a very high concentration, it is difficult to decide whether this agent acts as an adaptive enzyme inhibitor or by other mechanisms.

### Exp. 3. Influence of aeration, bubbling with oxygen and nitrogen gas, upon the induction of the adaptive enzymes.

In the secondary adaptation procedure, we encountered remarkable differences in induction of the adaptive enzymes between the cotton plugged shaking culture (100 cc medium in 500 cc Sakaguchi flask) and the culture aerated by mean of a compressor through a narrow glass tube in about 3l. of heavy suspension of cells (10g/cc wet cell) in a large glass vessel. In both cases the inductions were carried out for 3 hours at 30°C.

The former method resulted in a much higher adaptive enzyme formation than the latter method.

Hence, the influence of various ways of supplying oxygen and anaerobic conditions upon the induction of the adaptive enzymes were examined. A cell suspension of the same concentration as described in the standard secondary adaptation procedure was put in a shaking flask and incubated for adaptation in the following four ways. The bubbling was carried out through narrow glass tubes inserted through gum stoppers.

a) Oxygen gas bubbled for one and a half hours followed by aeration by means of a compressor. The rate of oxygen bubbling was 250 l. per flask per hour.

b) Vigorous aeration by means of a compressor.

c) Nitrogen gas was bubbled throughout the adaptation period. The rate of nitrogen bubbled was 80 l. per flask per hour.

d) As a control, the standard secondary adaptation procedure in a cotton plugged Sakaguchi flask was undertaken. After one and a half hour's and three hours's adaptation period, the adapted cells were harvested and measured for the induced adaptive enzymes, i.e., *m*-hydroxybenzoic acid oxidase and gentisase, by Warburg's method. The results obtained are shown in Fig. 8, 9, 10, 11. Differences between of the adaptive enzyme formation among a, b, and d, were not manifest, however, under nitrogen gas bubbling, i.e., under anaerobic conditions, adaptive formation of the gentisase and *m*-hydroxybenzoic acid oxidase did not occur at all. Therefore oxygen is considered to be necessary for the induction of the adaptive enzymes for these substrates by the organism.

### *Discussion*

Two kinds of contradictory results were reported so far on the relationship between adaptability of the cells and the age of the culture. One is, as reported by C.P. Hegarty,[2] that in the induction of galactozymase and lactozymase by *Streptococcus lactis* the physiologically young cells show the strongest adaptability. The other is, as reported by M.J. Pinsky,[3] that in the induction of formic hydrogenlyase by *E. coli* the adaptability steadily decreases during the period of active growth from the high level of the original inoculum and is restored as the cells enter the stationary phase of growth.

The results obtained by us are similar to the latter case. It may be said that for the adaptive enzymes which are essential for the metabolism of bacteria in their active growth phase, the cells in the log phase have strong adaptability, whereas, for the enzymes such as gentisase or *m*-hydroxybenzoic acid oxydase, which are usually not active in the course of active growth of bacteria, the cells in stationary phase have the strongest adaptability.

Therefore culturing the cells for eighteen hours, according to our procedure in the present study, is considered to be suitable.

Inhibition by streptomycin was used as one criterion to decide whether the enzyme in question is induced by the inducer substrate or the enzymes in question is one of the so called constitutive enzymes. As the induction of the enzyme was remarkably inhibited by the addition of streptomycin at the starting point, in the present study, it was further demonstrated that the gentisase and *m*-hydroxybenzoic acid oxidase were so called adaptive enzymes. However, it still remains to be shown whether the addition of the inhibitor after the induction of the enzyme has occurred actually does not suppress further induction of the enzyme as the analysis is very difficult. Suda et al[4] reported that induction of the adaptive enzymes did occur under anaerobic conditions i.e., in anaerobic adaptation of *Pseudomonas* in the conversion of histidine to glutamic acid, but in our case oxygen is necessary for the induction of the enzymes.

### *Summary*

Based on the fundamental conditions determined in the former part of this report on the adaptive enzyme formation of the nonproliferating intact cells in the secondary adaptation procedure, further investigations of the influence of the factors i.e. the age of the culture, method of supplying oxygen, and use of inhibitor against the induction of the adaptive formation of *m*-hydroxy-benzoic acid oxidase and gentisase, were examined and the following results were obtained.

1. The growth courve of *Pseudo-*

*monas ovalis S-5,* on both bouillon and bouillon glucose medium in shaking culture were obtained.

2. The adaptability of the intact cells prepared from the lag phase, biologically young phase, and log phase were weak and cells from the beginning to almost the end of the stationary phase exhibited the shortest lag time and highest $QO_2$ value. The old cells were very weak in adaptability.

3 Among the different methods of supplying oxygen in the course of the secondary adaptation procedure, i.e. oxygen gas bubbling, vigorous aeration by a compressor, and aeration through an ordinary cotton plug in shaking culture, no remarkable differences between them were observed in the rate of adaptive enzyme formation of both enzymes. Nitrogen gas bubbling, that is anaerobic conditions, completely supressed the formation of adaptive enzymes.

4. Streptomycin at a concentration of $10^{-4}$ M and $10^{-3}$ M a slightly higher concentration than the minimum inhibitory concentrotion of this organism, when added at the start of adaptation, completely suppressed the enzyme formation, however, once initatiaton of the adaptive enzyme formation had begun, streptomycin does not exhibit any inhibitory activity at all towards the formation of enzymes or towards the activities of the two enzymes.

The authors wish to express their sincerest thanks to Prof. K. Sakaguchi for his guidance and encouragement throughout this research, and also wish to thank Assist Prof. H. Takahashi for his useful discussions. We also wish to thank to Dr. H. Shimizu for the sample of gentisic acid.

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# 11. *Studies on the Carbohydrates of Soybeans.*

## V. *A Hemicellulose Fraction Soluble in Hot Water. Isolation and Component Sugar Determinations.*

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and Masahiro MINO

Dehulled and defatted soybean meal was treated with 0.2 % sodium hydroxide to give a sugar-free protein-extraction residue, from which hot-water-soluble hemicellulose was precipitated by adding ethanol to the concentrated hot-water extract. The hemicellulose was purified through the copper complex formed by adding Fehling's solution and acetone. Its decomposition point was 194∼5° and $[\alpha]_D^{20}$ +97.5° and it consisted of pentoses (arabinose and xylose) 17.8% (as pentosan), uronic acid (galacturonic acid) 19.2% (as lactone), and hexose (galactose) 41.9% (as galactan). The methods of analyses are described in detail.

### *Introduction*

In the manufacture of soybean protein[1,2] two by-products are produced, one of which, the waste liquor[3] of protein precipitation, or "soybean whey" as it is called in the U.S.A.[4], contains sucrose, raffinose, and stachyose with the monosaccharides produced from them by hydrolysis[5]. Soybeans or defatted soybeans contain only a trace of reducing sugar[6]. The contents of the three above-mentioned oligosaccharides were determined by Kawamura[7].

The other by-product obtained during the manufacture of soybean protein is the insoluble residue from protein extraction[8,9,10]. This residue contains sparingly soluble polysaccharides of soybeans. The separation of hemicelluloses from this residue by the method of Buston[11] was critically examined.[12] The result showed that hemicelluloses A and B increased only slightly by repeating the extractions with 4 % sodium hydroxide, while hemicellulose C increased successively by repeating the extractions and especially abruptly upon extraction at 98°. Thus it was presumed that a polysaccharide insoluble in water or in very dilute alkali (such as 0.2% sodium hydroxide) but soluble in hot water was present in the protein-extraction residue and was admixed in the fraction of hemicellulose C.

The authors separated and studied this polysaccharide fraction soluble in hot water. It may nearly correspond with the "pectin-like substance" mentioned by Sasaki[13], and was considered to belong to hemicelluloses (Whistler and Smart[14] defined hemicelluloses as water-insoluble cell wall polysaccharides of land plants except cellulose and pectin). It is called hot-water-soluble hemicellulose in this paper.

This study was made possible partly by the grant of the Ministry of Education (Scientific Research Fund) to Kawamura (studies on soybean hemicelluloses, 1951). Most parts of the experiments were carried out in the Laboratory of Biological Chemistry, Faculty of Agriculture, University of Tokyo, through the courtesy of Professor Yoshikazu Sahashi, Professor Yoshihiko Matsuyama, and Assistant-Professor Bunji Maruo. We wish to express our sincere thanks to them. Some later experimental works were made in the Laboratory of Biological Chemistry, Kagawa Agricultural College. Our thanks are due to Dr. Taiji Kurokami, the Director of Kagawa Agricultural College for his encouragement.

The outline of the study was presented (excluding the data described in paragraphs 5, 6 and 9-2 below) before the annual meeting of the Agricultural Chemical Society of Japan on April 7, 1953, at the Faculty of Agriculture, University of Tokyo.

## *Experimental*

### 1. Preparation of the protein-extraction residue of soybeans.

Defatted soybean flakes (obtained by low-temperature extraction with petroleum naphtha)[15] were pulverized to pass a 24-mesh sieve and were separated almost entirely from the seed hulls. About 2.8kg of dehulled defatted soybean meal (with 8.77 % moisture) was obtained. To this 28 l of 0.2% sodium hydroxide was added and the mixture was allowed to stand for one hour.

Table I.

The properties of crude (III) and purified (IV) hot-water-soluble hemicellulose.

| Tests | Crude (III) | Purified (IV) | |
|---|---|---|---|
| 1-Naphthol reaction | + | + | Carbohydrate |
| Anthrone reaction | + | + | Carbohydrate |
| Ag mirror test | − | − | Reducing power |
| Reduction of Fehling soln. | − | − | Reducing power |
| Seliwanoff test | − | − | Ketose |
| Phloroglucinol test | + | (+) | Pentose |
| Resorcinol test | + | (+) | Pentose |
| Aniline test | + | + | Pentose |
| Orcinol test | + | + | Pentose |
| Naphthoresorcinol test | + | + | Uronic acid |
| Basic Pb acetate test[16] | ... | + | Galacturonic acid |
| Oxidation to mucic acid | + | + | Galactose and/or galacturonic acid |
| Acetone test | − | − | Methylpentose |
| Pptn. as Ca salt | − | − | Pectic acid |
| Iodine reaction | + | − | Starch |
| Melting point (decompn.) | 191∼4° | 194∼5° | |
| $[\alpha]_D^{20}$ | ... | +97.5° | |
| Nitrogen | ... | 0.17 % | |
| Ash | ... | 3.1 % | |
| $OCH_3$ | 0.68 % | ... | |

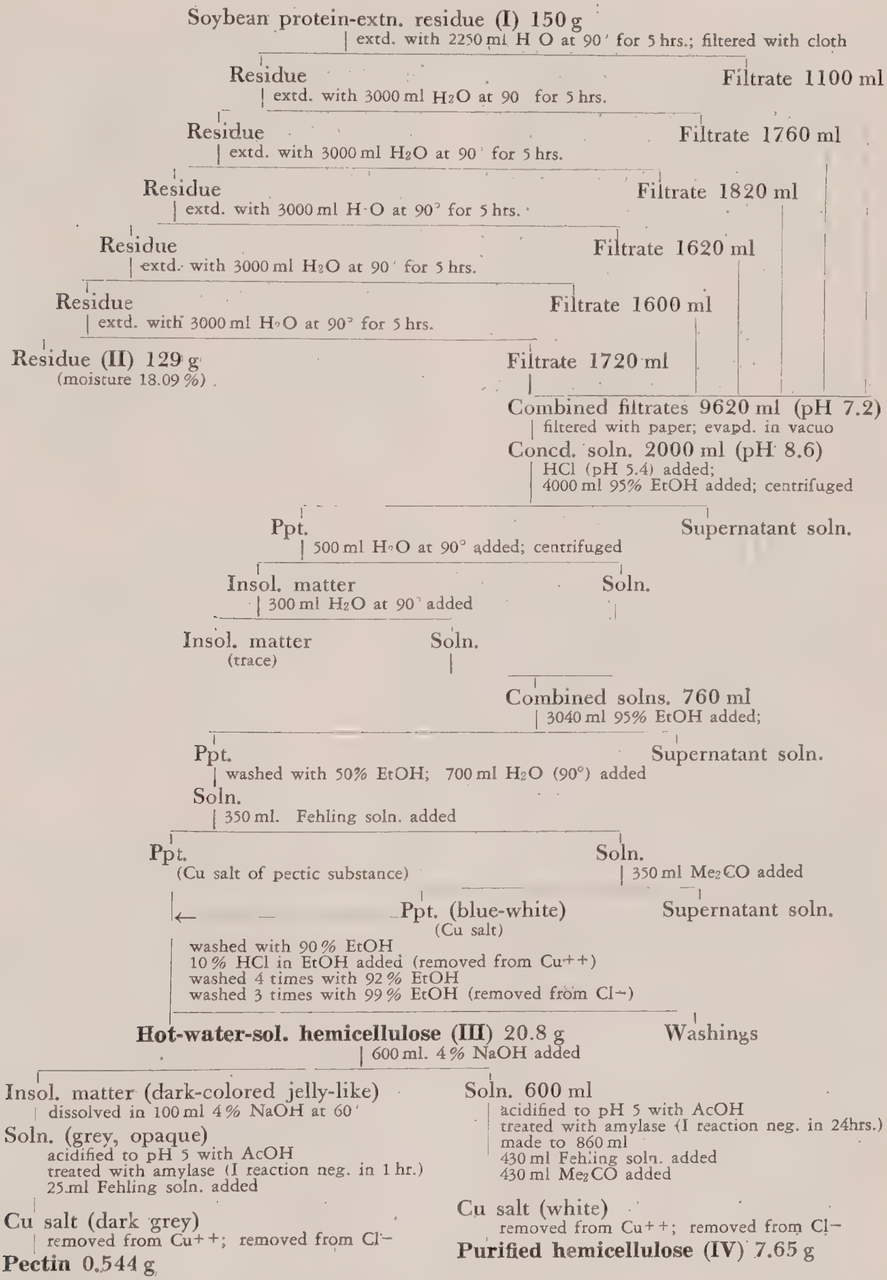
Soybean protein-extn. residue (I) 150 g
extd. with 2250 ml $H_2O$ at 90° for 5 hrs.; filtered with cloth
Residue
Filtrate 1100 ml
extd. with 3000 ml $H_2O$ at 90° for 5 hrs.
Residue
Filtrate 1760 ml
extd. with 3000 ml $H_2O$ at 90° for 5 hrs.
Residue
Filtrate 1820 ml
extd. with 3000 ml $H_2O$ at 90° for 5 hrs.
Residue
Filtrate 1620 ml
extd. with 3000 ml $H_2O$ at 90° for 5 hrs.
Residue
Filtrate 1600 ml
extd. with 3000 ml $H_2O$ at 90° for 5 hrs.
Residue (II) 129 g
(moisture 18.09 %)
Filtrate 1720 ml
Combined filtrates 9620 ml (pH 7.2)
filtered with paper; evapd. in vacuo
Concd. soln. 2000 ml (pH 8.6)
HCl (pH 5.4) added;
4000 ml 95% EtOH added; centrifuged
Ppt.
Supernatant soln.
500 ml $H_2O$ at 90° added; centrifuged
Insol. matter
Soln.
300 ml $H_2O$ at 90° added
Insol. matter
(trace)
Soln.
Combined solns. 760 ml
3040 ml 95% EtOH added;
Ppt.
Supernatant soln.
washed with 50% EtOH; 700 ml $H_2O$ (90°) added
Soln.
350 ml. Fehling soln. added
Ppt.
(Cu salt of pectic substance)
Soln.
350 ml $Me_2CO$ added
Ppt. (blue-white)
(Cu salt)
Supernatant soln.
washed with 90 % EtOH
10 % HCl in EtOH added (removed from $Cu^{++}$)
washed 4 times with 92 % EtOH
washed 3 times with 99 % EtOH (removed from $Cl^-$)
Hot-water-sol. hemicellulose (III) 20.8 g
Washings
600 ml. 4 % NaOH added
Insol. matter (dark-colored jelly-like)
dissolved in 100 ml 4 % NaOH at 60°
Soln. (grey, opaque)
acidified to pH 5 with AcOH
treated with amylase (I reaction neg. in 1 hr.)
25 ml Fehling soln. added
Cu salt (dark grey)
removed from $Cu^{++}$; removed from $Cl^-$
Pectin 0.544 g
Soln. 600 ml
acidified to pH 5 with AcOH
treated with amylase (I reaction neg. in 24hrs.)
made to 860 ml
430 ml Fehling soln. added
430 ml $Me_2CO$ added
Cu salt (white)
removed from $Cu^{++}$; removed from $Cl^-$
Purified hemicellulose (IV) 7.65 g

Fig. 1. The procedure of isolating the hot-water-soluble hemicellulose from the soybean protein-extraction residue.

After filtration the residue was treated with another batch of 0.2 % sodium hydroxide (28 l) for one hour. The residue, washed twice with water and dried under the sun, gave 402g of protein-extraction residue (with 8.22% moisture). The treatment with 0.2% sodium hydroxide is effective in solubilizing most of the protein and all of the sugars present[1,8]. The yield of the protein-extraction residue amounted to 14.5% of the defatted meal.

### 2. Isolation of hot-water-soluble hemicellulose.

The protein-extraction residue was extracted with hot water (repeated six times), and the hot-water-soluble extract was evaporated under reduced pressure. Two volumes of 95% ethanol were added to obtain a precipitate, which was dissolved in hot water, precipitated as the copper salt with Fehling's solution and acetone, and freed from the copper ion. The procedure is shown in Fig. 1.

### 3. Qualitative and quantitative tests on the hot-water-soluble hemicellulose.

The polysaccharide **III** was contaminated with a substance giving a positive reaction (blue) to the starch-iodine test. The purified sample **IV** was free from it. The result of the tests on **III** and **IV** is summarized in Table I.

### 4. Reducing power of the hydrolyzate of hot-water-soluble hemicellulose.

The Somogyi method[17] was applied. (*a*) Preliminary experiments with pure sugars showed that 1 ml of 0.005 N sodium thiosulfate corresponded with 0.133 mg glucose, 0.139 mg arabinose, 0.157 mg galactose. (*b*) In the first experiments 5.9 mg of the hot-water-soluble hemicellulose **IV** was placed into 5 tubes containing 5 ml N hydrochloric acid and the mixture was kept at 100° for 2, 4, 6, 8 and 10 hours. The hydrolyzate after neutralization was analyzed for reducing sugar. The result expressed as galactose is shown by curve I of Fig. 2. (*c*) In the second experiments hydrolysis of 6.15 mg hemicellulose **IV** with 5 ml N hydrochloric acid was carried out in the same way as above for 1, 2, 3, 4 and 5 hours. The result is shown by curve II of Fig. 2.

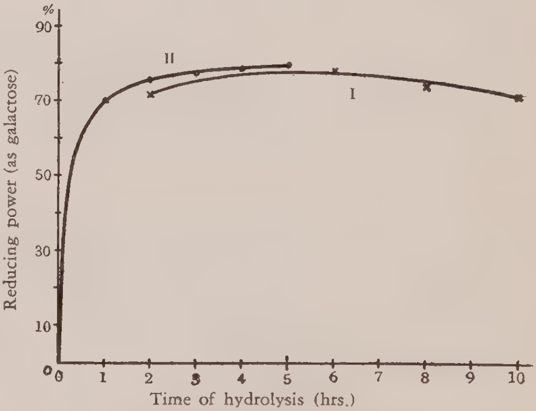

Fig. 2.

### 5. Determination of uronic acid.

The method of Dickson et al.[18] was applied with success. The result is shown in Table II.

### 6. Determination of furfural yield.

The Tollens distillation method was applied. The experimental data are shown in Table III.

### 7. Determination of total "galactose."

The method of Van der Haar[19] was applied. Preliminary experiments with pure galactose showed that the recovery was 89.9%. When 0.3874g of sample

Table II

Determination of uronic acid.

| Sample,[1] g | Titration, ml | Uronolactone[2], g | % | Average |
|---|---|---|---|---|
| 0.4737 | 10.86 | 0.09164 | 19.34 | 19.2 % |
| 0.4386 | 9.96 | 0.08338 | 19.01 | |

Note 1: The sample was dried in vacuo over phosphorus pentoxide for 16 hours.

Note 2: Uronolactone (g) = 0.0022 × titration (ml) × 0.9589 × 4, where 0.9589 is the factor of HCl soln.

Table III

Determination of furfural yield.

| | |
|---|---|
| Sample | 0.3387 g |
| Furfural phloroglucide | 0.0953 g |
| Furfural phloroglucide to be formed from galacturonolactone[1] | 0.0272 g |
| Furfural phloroglucide to be formed from pentose | 0.0680 g |
| Pentosan | 0.0603 g or 17.8 % |

Note 1: 0.3387 g × 0.1918 (see Table II)/2.39 = 0.02718 g, where 2.39 is the factor for converting phloroglucide to galacturonolactone[24)].

**IV** dried with the Abderhalden drier for 5 hours was added with 60 ml nitric acid (d = 1.15) and the mixture was concentrated to 20 g, a little dark green precipitate was formed. (This precipitate removed and dried weighed 0.018 g or amounted to 0.46% of **IV**; its nature was not examined.) After filtration of this minute amount of precipitate, the filtrate and washing (with water) were combined and concentrated to 20 g. Mucic acid (0.5101 g) was added and the mixture was allowed to stand for 48 hours with intermittent stirring and rubbing of the internal wall of the beaker. The crystals of mucic acid weighed 0.6870 g after filtration, washing, and drying. Thus the "galactose" content was (0.6870 − 0.5101)/0.3874 × 100/89.9 = 70.6%.

### 8. Determination of pentose and galactose.

Albaum and Umbreit[20)] reported the method to determine separately various pentoses and pentose phosphates based upon different degrees of the coloration in the orcinol pentose reaction. Drury[21)] extended this method to determine colorimetrically pentoses and glucose. Under certain conditions glucose and pentose behaved

Table IV

Colorimetry of the hydrolyzed sample by the Drury method.

| Sample, $\gamma$ | 41.1 | 51.4 | 82.3 | 123.4 | Average |
|---|---|---|---|---|---|
| Reading *R* | 0.069 | 0.098 | 0.173 | 0.274 | |
| Reading *G* | 0.039 | 0.052 | 0.088 | 0.148 | |
| Arabinose, % | 7.88 | 11.1 | 14.2 | 12.5 | 11.4 |
| Galactose, % | 70.2 | 72.5 | 74.5 | 86.6 | 75.9 |

Note: *R* and *G* are the readings of the mixture of the sample and the reagents with red (660 mμ) and green (540 mμ) filters, respectively. For calculation, see the reference[25)].

differently in the orcinol reaction, and the colorimetric readings at intervals gave the pentose content within a 2% error after suitable mathematical treatment. We have determined arabinose and galactose by the Drury method with the result as shown in Table IV. The details of this method are described elsewere[25].

### 9. Detection of component sugars by paper chromatography.

**9.1. Detection of xylose, arabinose, and galactose.** About 8 ml of the solution (containing 12.34 mg of sample hemicellulose **IV** per 10 ml) was heated with 10 ml N hydrochloric acid in a boiling-water bath for 5 hours. The acid hydrolyzate was neutralized by mixing with the resin Amberlite IRA 400 (previously activated with 0.5N sodium hydroxide). The filtrate at pH 7.0 was concentrated in vacuo to about 1 ml. This was kept in a test tube with absolute alcohol. The concentration of sugar was about 0.96%. The result of chromatography is shown in Table 5. The technique used was the one-dimensional ascending method. The developing reagent used was aniline hydrogen phthalate. The spot of galactose was brown, while the spots of xylose and arabinose were pink. The spot of the sample corresponding to galactose was always more strongly colored than the pentose spots.

**9.2. Detection of uronic acid as well as the three sugars.** About 0.01 g of the sample hemicellulose **IV** was heated in sealed tubes with 0.2ml 4% sulfuric acid. After 1, 6, 12 and 24 hours the acid hydrolyzate was directly chromatographed on paper. The one-dimensional ascending technique was used with anisidine hydro-

Table V

Paper chromatograms of the hydrolyzate of hot-water-soluble hemicellulose. (9–1)

| Solvent | BuOH-AcOH-$H_2O$ (2 : 4 : 1) | | BuOH-pyridine-$H_2O$ (6 : 4 : 5) | | | | PhOH-$H_2O$ (85 : 15) | |
|---|---|---|---|---|---|---|---|---|
| | S. | H. | S. | H. | S. | H. | S. | H. |
| Xylose | — | — | 0.430 | 0.447 | 0.473 | 0.457 | 0.592 | 0.592 |
| Arabinose | 0.152 | 0.158 | 0.362 | 0.380 | 0.409 | 0.396 | 0.740 | 0.719 |
| Galactose | 0.078 | 0.083 | 0.282 | 0.291 | — | 0.306 | 0.545 | 0.536 |

Note: S.: $R_f$ for standard sugars. H.: $R_f$ of spots from hemicellulose.

Table VI (a, b, c)

Paper chromatograms of the hydrolyzates of hot-water-soluble hemicellulose. (9–2)

Solvent: BuOH–AcOH–$H_2O$ (5 : 2 : 3)

| Corresponding sugar | Color of spot | (a) | (b) | (c) |
|---|---|---|---|---|
| Pentose | Red | 0.41 | 0.37 | .... |
| Galactose | Yellow | 0.33 | 0.295 | .... |
| Uronic acid | Red | 0.28[1] | 0.25 | 0.25 |
| Aldobiuronic acid (?) | Faint | .... | .... | 0.145[2] |

Note 1: This spot appeared later; the duration of hydrolysis and the degree of the color intensity were, resp., 1 hr., –; 6 hrs., +; 12 hrs., + +; 24 hrs., + +; while the other 2 spots always gave a higher coloration (+ + +).

Note 2: This spot appeared faintly only after 24 hrs.

Table VI (d)

| Solvent | BuOH–AcOH–$H_2O$ (4 : 1 : 5) S. | H. | PhOH–$H_2O$ (5 : 1) S. | H. |
|---|---|---|---|---|
| Galactose (yellow) | 0.24 | 0.24 | 0.423 | 0.423 |
| Arabinose (red) | 0.288 | 0.31~.32 | 0.535 | 0.538 |
| Xylose (red) | 0.32 | | 0.450 | 0.457 |

Note: S.: $R_f$ for standard sugars. H.: $R_f$ of spots from hemicellulose.

chloride as the developing reagent. The chromatogram is shown in Table 6 (a). The rest of the hydrolyzate was combined. After neutralization with barium carbonate (b), 4 parts of ethanol were added to obtain the precipitate (c) or acid sugar fraction and the soluble part (d) or neutral sugar fraction. The chromatograms of (b) (neutralized hydrolyzate), (c), and (d) are shown in Table VI (b, c, d). Thus the hydrolyzed sample contained aldobiuronic acid (?) ($R_f$ 0.145 with butanol-acetic acid-water (5 : 2 : 3)) (cf. Mino[22]), uronic acid, galactose, arabinose, and xylose.

### *Discussion*

In isolating this polysaccharide, a fraction was obtained which is assumed to be pectin or a pectic substance (Fig. 1) in the amount of 0.544g from 150 g of soybean protein-extraction residue **I** or 0.40%. This was not further examined. The isolation of the pectic substance from dehulled soybeans has not yet been recorded in the literature.

The yield of hot-water-insoluble residue **II** was 76.7 % of protein-extraction residue **I.** This should contain other hemicelluloses and cellulose. The yields of crude (**III**) and purified (**IV**) hot-water-soluble polysaccharide samples were 15.1% and 5.54%, respectively, of protein-extraction residue **I.** The chief difference between the samples **III** and **IV** concerns the contamination with starch or a similar polysaccharide giving a blue coloration with iodine. The starch present in a minute amount in soybeans[23] does not give a positive iodine reaction when tested directly on soybeans or defatted soybeans. However, the crude polysaccharide **III** gave a starch-iodine reaction, evidently because starch was completely freed from the interfering substance such as fatty acids.

The hot-water-soluble hemicellulose had no reducing power; it contained pentoses (arabinose and xylose), galactose, and galacturonic acid, but no ketose or methylpentose (Tables I, V, and VI). The detection of xylose in the hydrolyzed sample was not expected and it is the first time that xylose was found as a constituent of soybean carbohydrates.

Concerning the condition of hydrolysis, this hemicellulose gave maximum reducing power when heated with N hydrochloric acid for 5 hours (see curve II of Fig. 2). Curve I of Fig. 2 shows that it lost some of its reducing power with N hydrochloric acid upon heating longer than 5 hours. This may be due to the presence of pentoses and uronic acid, which are liable to decomposition.

The analyses showed that this hemicellulose consisted of pentoses (arabi-

Table VII

Calculation of true galactan content.

| | As detd. | Equiv. mucic acid calcd. | Calcd. |
|---|---|---|---|
| Total "galactose" | 70.6 | 52.2[1] | |
| Galacturonic acid | 21.1[2] | 17.3[3] | |
| True galactose | | 34.9 | 46.5 4) as galactose<br>41.5 5) as galactan |

Note 1: 70.6/1.33 = 52.2.
Note 2: 19.2 (as detd.) × 194 (M.W. of $C_6H_{10}O_7$)/176 (M.W. of $C_6H_8O_6$) = 21.1.
Note 3: 21.1 × 82.2/100 ≒ 17.3, where 82.2 ≒ 210 (M.W. of $C_6H_{10}O_8$)/194 (M.W. of $C_6H_{10}O_7$) × 0.76; since only 76% of theory can be recovered as mucic acid from galacturonic acid.24)
Note 4: 34.9 × 1.33 = 46.5.
Note 5: 46.5 × 0.90 = 41.9.

nose and xylose), galactose, and galacturonic acid after hydrolysis. The true galactose or galactan content can be calculated from the value of "galactose" which is determined by oxidation to mucic acid (70.6%, paragraph 7) and the value of galacturonolactone (19.2 %, Table I). This correction is necessary, since not only galactose but also galacturonic acid gives rise to mucic acid by oxidation. As shown in Table VII, the true galactan content is 41.9%.

The result of colorimetry by the Drury method (11.4% pentose and 75.9% galactose) (Table IV) is not reliable, since the hydrolyzed hemicellulose contains not only pentoses and hexose but also hexuronic acid.

In conclusion this hemicellulose consists of 17.8% pentoses (as pentosan), 19.2% galacturonic acid (as lactone), and 41.9 % galactose (as galactan), the pentoses being arabinose and xylose.

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## 12. *Studies On Cycasin, a New Toxic Glycoside, of Cycas revoluta Thunb.*

### *Part 1. Isolation and the Structure of Cycasin.*

By Kotaro NISHIDA, Akira KOBAYASHI and Tomonori NAGAHAMA

Cycasin, a new toxic glycoside, $C_8H_{16}O_7N_2$, has been isolated from the seeds of Japanese cycad, ion-exchange resins and active carbon chromatography being used. From the results of the examination of the decomposition products such as nitrogen gas, formaldehyde, and cyanide etc. and of the ultraviolet and infrared absorption spectra, its aglycone is considered to be identical with that of macrozamin from Australian cycad, reported by Lythgoe et al. It is found, however, that the sugar component of cycasin is one molecule of glucose, unlike that of macrozamin namely primeverose. Cycasin is, therefore, concluded to be a new compound, glucosyloxyazoxymethane.

It has been well known that some poisonous component is contained in the kernels of the seeds of Japanese cycad, *Cycas revoluta* Thunb., which grows wildly in the southernmost Japan and in the District of Ryukyu. Proving the existence of formaldehyde in these kernels, Yoshimura[1)] suggested that it would be the toxin itself. One of the authors, Nishida,[2,3)] then confirmed that it should be the enzymatic decomposition product of a $\beta$-glycoside which was predicted by him in the consequence of the studies on the relation with cycad-emulsin.

Recently, Lythgoe et al.,[4,5)] studied the structure of a new toxic glycoside which was first isolated from Australian cycad, *Macrozamia spiralis*, and was named macrozamin by Cooper.[6)] According to them macrozamin contains linked nitrogen atoms which have not previously been found in natural product. Moreover, Riggs[7)] reported that the toxic component was macrozamin in every one of eight species of Australian cycad examined.

Succeeding the prewar studies on Japanese cycad, the authors successfully isolated the predicted glycoside in a crystal state by the chromatographic removement of impurities, especially sugars, which interfered with the crystallizing. It being presumed that the aglycone of cycasin would be an analogous structure to macrozamin, the chemical behaviors and absorption spectra of cycasin were examined to compare with those reported by Lythgoe et al.[5)] It was consequently clarified that though they have an aglycone of identical structure, they decisively differ from one another in sugar component.

## *Experimental*

### A. Isolation of Cycasin.

The process is shown in Table I.

## Table I

### Isolation of Cycasin

Seeds 14 kg
|—hulled, boiled immediately for 20 min.
|—minced, dried, and milled

Powder of Kernels 5 kg
|—extracted with 20 l of cold $H_2O$, repeated three times
|—filtered by cloth

Filtrate 60 l | Residues
—vac. condensed at 60°C
|—added 3 vols. EtOH, filtered

Filtrate 1.8 l | Ppts.
| vac. condensed at 60°C

Syrup A 1.3 l
|—passed through column of Organolite-K, repeated till the solution becomes colorless (pH 1.2)
|—passed through column of Amberlite IR-4B, repeated till pH ascends to 5.8
|—vac. condensed at 60°C

Syrup B 500 cc
|—adsorbed on active carbon column
eluted with $H_2O$

Carbon Column | Aqueous Eluate 1.8 l
|—eluted with 10% EtOH
|—chromatographied with carbon, as Syrup B (repeated several times)

Ethanolic Eluate 2 l
|—condensed to thick syrup
—added 3 vols. EtOH
—set aside in refrigerator

Cycasin | Aqueous Eluate
|—Glucose
|—Fructose
|—Sucrose etc.

Cycasin 7.5 g

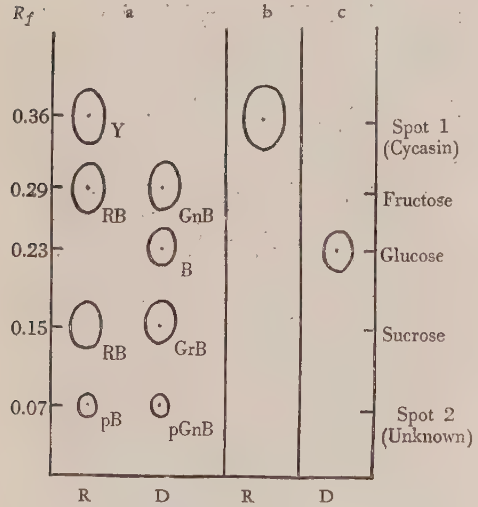


Fig. 1. Paper Chromatograms of Cycasin and Sugars.

Sample:—a; Syrup A and B, b: Crystal of Cycasin, d; Hydrolysate of Cycasin
Developing Solvent:—BuOH, AcOH, $H_2O$ Mixture (4:1:1)
Spray Reagent:—R; 1% Resorcin Soln. in EtOH, 2N HCl Mixture (1:9), D; 2% Diphenylamine, 5% Trichloroacetic Acid Soln. in BuOH, MeOH Mixture (1:1)
Color:—Y; yellow, R; red, B; brown, Gr; grey, Gn; green, p; pale

Enzymes in the kernels were inactivated by the treatment with boiling water. Starch and sticky materials were precipitated by the addition of alcohol to the aqueous extract, and then electrolytes such as organic acids and bases and inorganic salts etc. were removed by passing through the columns of ion-exchange resins. The sugars contained in the Syrup B were paper chromatographically identified as glucose, fructose, and sucrose (Fig. 1), which were the same as those found in Syrup A. The Spot 1 in Fig. 1 was assumed to be that of the glycoside, since the possibility of it being that of a methylpentose, suggested by its color,[8] should be eliminated because of its *Rf* value or because of the negative reaction by acetone-HCl method, and since this spot was not observed in the extract of non-boiled kernels or when the Syrup B was boiled with acid.

The separation of the glycoside from sugars was carried out by the carbon chromatography, the method of Whistler et al.[9] being modified. The column of 100 g of active carbon in a 5 (diameter) × 25 cm glass cylinder was used. After glucose, fructose, and sucrose which was most difficult to remove, were eluted with about 1.45 l of water from the column as shown in Fig. 2, the remaining glycoside was eluted out with about 2 l of 10% alcohol. The treatment with carbon column was repeated several times on the aqueous eluate to recover as much cycasin as possible.

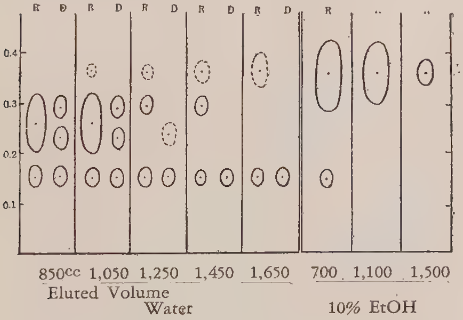


Fig. 2. Elution of Sugars and Cycasin from the Active Carbon Column, shown in Paper Chromatograms (Development and Reagent are the same as in Fig. 1)

## B. Properties of Cycasin.

The following properties were observed on the crystal of cycasin thus obtained:—Soluble in water or dilute alcohol, sparingly soluble in pure alcohol, and insoluble in benzene, acetone, chloroform, or ethyl acetate. Not precipitated by neutral or basic lead acetate. Molish reaction was positive and the coloration with anthrone was greenish blue. On paper chromotograms it gave single spot, which was taken the place by that of glucose after acid hydrolysis (Fig. 1-b, c). Colorless needle crystal (Fig. 3), m.p. 144°~145° (decomp), $[\alpha]_D^{28} = -41.3°$ (c, 2.0, water). *Anal.* Found: C, 38.17; H, 6.37; N, 10.93, Calcd. for $C_8H_{16}O_7N_2$: C, 38.07; H, 6.39; N, 11.11%. MW, Found: 253 (freez. point). Calcd.: 252.

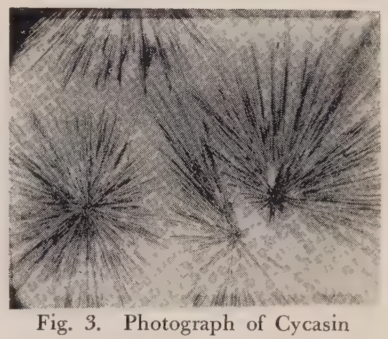


Fig. 3. Photograph of Cycasin

Acetyl derivative of cycasin prepared by the usual manner with pyridine and acetic anhydride was a white powder, which was crystallized in the rectangular form (Fig. 4) when treated with boiling water and allowed to cool. -30
M.p. 137°~138°, $[\alpha]_D^{20} =$ ~~−4.0~~° -25.4° (c, 0.6, $CHCl_3$). *Anal.* Found: C, 45.75; H, 5.70; N, 6.90, Calcd. for $C_{16}H_{24}O_{11}N_2$: C, 45.72; H, 5.75; N, 6.66%. MW, Found: 409 (Rast), Calcd.: 420.

-25.4°

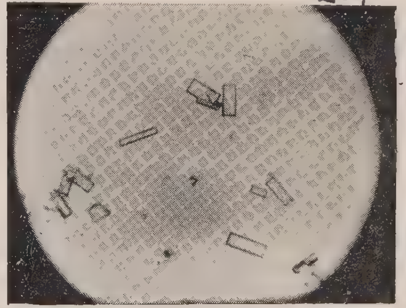


Fig. 4. Photograph of Acetyl Cycasin

## C. Aglycone Part.

**I. Acid Hydrolysis.** When heated at 100° with N-hydrochloric acid, the aglycone of cycasin, being incapable of independent existence, was decomposed in nitrogen gas, formaldehyde, and methanol. The analytical results of these products are summarized in Table II, in which the data on macrozamin by Lythgoe are also shown for comparison.

Table II

The Comparison between the Decomposition products of Cycasin and Those of Macrozamin

| | Decomposition Products | Per Mol. of Cycasin | Per Mol. of Macrozamin* |
|---|---|---|---|
| | | Mol. | Mol. |
| Acid Hydrolysis | $N_2$ Gas | 0.92 | 1.02 |
| | Formaldehyde | 0.94 | 0.98 |
| | Methanol | 1.07 | 0.51 |
| Alkali Decomposition | $N_2$ Gas | 0.35 | 0.45 |
| | Formic Acid | 0.31 | 0.45 |
| | Cyanide | 0.37 | 0.52 |
| | Volatile Bases | Equiv.<br>0.17 | Equiv.<br>0.18 |

* cited from Lythgoe's report

1. Nitrogen Gas. A 20cc conical flask containing cycasin and full of N-hydrochloric acid was connected to a gas burette which was filled with air-free distilled water, and was heated in a boiling water bath for about three hours till gas evolution had ceased. The gas volume was measured after the apparatus was allowed to cool, and the usual analysis on this gas showed that it was nitrogen gas.

2. Formaldehyde. Cycasin was hydrolysed with N-acid under reflux at 100° for two hours. The solution was treated with dimedone, and the analysis of the obtained colorless small needle crystal agreed with that of formaldomedone as follows, m.p. 189°, *Anal.* Found: C, 70.08; H, 8.25, Calcd. for $C_{17}H_{24}O_4$: C, 69.83; H, 8.25%. The determination of formaldehyde was carried out on the neutralized hydrolysate according to the colorimetric method by Tanenbaum et al.[10)]

3. Methanol. Since the simultaneous formation of formaldehyde interfered with the measurement of methanol, the former was caught as domedone and filtered off from the hydrolysate preliminarily. The filtrate was distilled and methanol was determined colorimetrically by fuchsin-sulfite.[11)]

**II. Alkali Decomposition.** As the decomposition products of cycasin with N-sodium hydroxide solution, nitrogen gas, cyanide, formic acid, and volatile bases were obtained. The data are shown in Table II.

1. Nitrogen Gas. The same procedures as indicated in the acid hydrolysis were adopted, where N-acid was substituted with N-sodium hydroxide solution.

2. Formic Acid. The resultant solution in the estimation of nitrogen gas was transferred quantitatively to the Pozzi's micro apparatus, and was steam distilled after being acidified with phosphoric acid. The distillate was titrated with N/50 sodium hydroxide solution. The neutral solution so ob-

tained, reduced remarkably ammoniacal silver nitrate, and this result indicated that formic acid was formed.

3. Cyanide. The solution similarily decomposed was acidified and then steam distilled. The distillate was titrated with N/50 silver nitrate solution.[12]

4. Volatile Bases. Cycasin was digested with N-sodium hydroxide solution in a micro Kjeldahl apparatus, and then the steam distillate was evaporated after being titrated with standard hydrochloric acid. Hydrochlorides of the bases were developed on filter paper with N-butanol, acetic acid, water mixture (4:1:1, esterified completely at room temperature), and were detected with 0.2% bromophenolblue solution in absolute alcohol[13] as two yellow spots of *Rf* values 0.11 and 0.19 respectively. The former was identical with that of an authentic specimen of ammonium chloride, and the latter, methylamine hydrochloride. Moreover, these two bases were assumed, from the area and color intensity of the spots, to be a mixture of approximately equal amounts.

**III. Action of Stannous Chloride.** Cycasin (50 mg), stannous chloride (350 mg), and 5N-hydrochloric acid (5 cc) were taken in a test tube, which was vacuumed, sealed, and kept at room temperature for seven days. A similar tube containing no cycasin was made as a control to confirm whether no aerial oxidation had occurred in these conditions. The results of iodometric titration showed that the consumption of stannous chloride by cycasin corresponded to 3.01 mols. per mol. of cycasin. The formation of volatile bases was proved to be 1.85 equivs., and that of formaldehyde 0.98 mol. per mol. of cycasin, when examined according to the procedures described above.

**IV. Absorption Spectra.** The ultraviolet absorption spectrum was measured on a 5/1000% aqueous solution of cycasin, using Shimadzu photoelectric spectrophotometer. In Fig. 5 the molecular absorption, log ε, is compared with that of macrozamin reported by Lythgoe. The infrared absorption spectra of cycasin and acetyl cycasin are shown in Fig. 6 along with those of macrozamin and acetyl macrozamin.

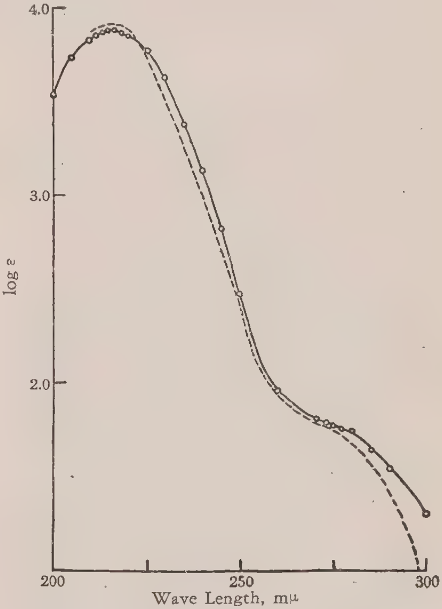


Fig. 5. Ultraviolet Absorption Spectra.
—— Cycasin
······ Macrozamin, duplicated from Lythgoe's report

**D. Sugar Component.**

Having observed by the paper chromatography described above that the sugar component of cycasin was glucose, it was further confirmed.

1. Glucosazone. The osazone of

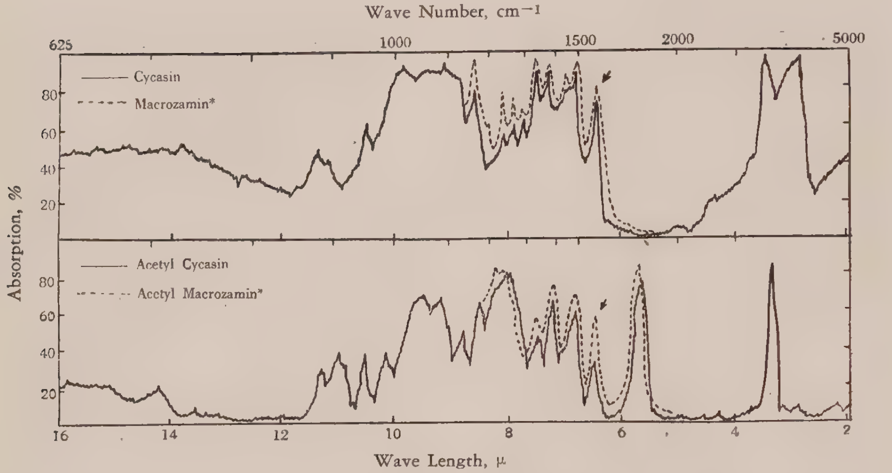


Fig. 6. Infrared Absorption Spectra.

Samples were examined as paste with nujol, to which the bands near 6.85 and 7.25μ are due. The band at ca. 1760 cm$^{-1}$ is due to the ester carbonyl groups. Marked spectra, *, are duplicated from Lythgoe's report.

the sugar component of cycasin was prepared after the usual treatment of the acid hydrolysate of cycasin. Its m.p. was 208°, undepressed on admixture with an authentic glucosazone.

2. Determination of Glucose. The acid hydrolysate of 20.2mg of cycasin was filled up to 50cc after formaldehyde being expelled by repeated vacuum evaporation. Determination was carried out on the aliquot by Hanes method, and 14.54mg of glucose was found (1.01 mols. per mol. of cycasin).

3. Survey of Xylose in the Kernels. Raw seeds (about 40 g) were hulled carefully and extracted with boiling methanol. The existence of cycasin was proved by paper chromatographic analysis on the condensed extract. Since the spot of xylose could not be found on the chromatograms, it may be concluded that xylose does not exist even in a free state.

### *Discussion*

A number of trials, to isolate cycasin directly in crystal form as shown in the case of macrozamin, were in vain. The difficulties, lying principally in the removal of the impurities, especially of a great amount of sugars, were resolved by the carbon chromatography. It could not, however, be expected to make the size of the column large enough to make the yield better, because the developing solvents scarcely passed through the column even in the size described above. The amount of cycasin which was separated in one round of chromatography was, therefore, very small, and even after the treatments was repeated several times, the yield was confined within only 0.05% of the raw seeds.

The equation for the acid hydrolysis of cycasin which is considered to have the constitution of $\text{Gl-C}_2\text{H}_5\text{O}_2\text{N}_2$, where Gl is the glucosyl residue, is shown as

$$\text{Gl-C}_2\text{H}_5\text{O}_2\text{N}_2+\text{H}_2\text{O}=\text{Glucose}+\text{N}_2+\text{HCHO}+\text{CH}_3\text{OH}$$

above from the quantitative data of each product.

The one carbon atom of the two in the aglycone appears as formaldehyde, and the other methanol. Two atoms of nitrogen are evolved quantitatively as molecular nitrogen gas. This result suggests strongly that cycasin would have linked nitrogen atoms in its molecule as macrozamin has. This evolution was also observed in the alkali decomposition. Nevertheless, features being more complex in this case than in acid hydrolysis, stoichiometric results were not obtained. The reduction of cycasin with stannous chloride is considered to follow the equation

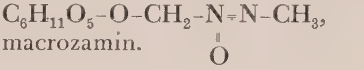
$$Gl{-}C_2H_5O_2N_2+3\,SnCl_2+6HCl=Glucose+3\,SnCl_4+CH_3NH_2+NH_3+HCHO$$

These chemical behaviors of cycasin agree with those of macrozamin, and thus it is concluded, as Lythgoe reported, that (a) the aglycone of cycasin has C–N=N system and (b) the sugar component is combined to the aglycone through nitrogen or carbon atom.

According to Lythgoe, the data on the properties and the ultraviolet absorption spectrum of macrozamin, eliminated the possibility of azo, nitrosamin, diazo, azoether, or nitrous oxide, except azoxy structure as the possible structure for it. Hence, he examined the ultraviolet and infrared absorption spectra of four aliphatic azoxy compounds such as ethyl-2-azoxybutyrate, and found that they have the same absorption mixima as macrozamin. Lythgoe et al.[14] have given further evidence to these facts with the synthesized simple aliphatic azoxy homologues.

The ultraviolet absorption curve of cycasin agrees closely with that of macrozamin, showing an eminent absorption maximum at 217 $m\mu$ and an inflexion at 275 $m\mu$. In the infrared absorption spectra, too, cycasin and acetyl cycasin show the same specific peak at 1540 $cm^{-1}$ as macrozamin. From these facts, it is apparent that cycasin has the chromophore, –N(O)–N–, of aliphatic azoxy group.

On the other hand, it is also clear that the sugar component of cycasin is only one molecule of glucose, and that the possibility should be denied that cycasin would have been obtained as a glycoside from which xylose was splitted during the isolation process.

It is consequently concluded that cycasin is glucosyloxyazoxymethane,  $C_6H_{11}O_5\text{-}O\text{-}CH_2\text{-}\underset{\underset{O}{\|}}{N}{=}N\text{-}CH_3$, unlike, macrozamin.

## *Acknowledgement*

The authors wish to express their sincere gratitude to professor Tetsuo Mitsui of Kyoto University for his kindly help on the elementary analysis, and also to Pharmacognostical Laboratory of Tokyo University for their kindness in measuring the infrared absorption spectra.

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# 13. *Biochemical Studies on* " Bakanae " *Fungus.*

## *Part 33. 5-Hydroxymethyl-furan-2-carboxylic acid.*

Akira KAWARADA, Nobutaka TAKAHASHI, Hiroshi KITAMURA,
Yasuo SETA, Makoto TAKAI and Saburo TAMURA

In the study on the chemical structure of gibberellins, the submerged culture method was applied to obtain a large amount of gibberellins. While isolating them from the medium, a crystal having m.p. 164–5° was obtained and identified to be 5-hydroxymethyl-2-furan-carboxylic acid.

The gibberellins, the metabolic products of *Gibberella fujikuroi*, having a very particular physiological activity, have been studied since 1939 in our laboratory, and thirty two reports in this series already have been published. The present paper deals with the 5-hydroxymethyl-furan-2-carboxylic acid as one of the metabolic products of the above fungus.

Since a large amount of gibberellins are necessary for the study of their chemical structure, the submerged culture method using a 600 l tank was carried out several times and 5-hydroxymethylfuran-2-carboxylic acid was isolated in pure crystals as a by-product of gibberellins. The identification was established from the results of analytical data, mixed melting point as well as its absorption spectra of infrared and ultraviolet rays.

This acid was first isolated from the cultured media of several fungi as a metabolic product by Sumiki[1,2].

### *Experimental*

### 1) Submerged culture.

*Gibberella fujikuroi* was cultivated at 27–29° for 72–90 hours in a stainless steel tank under airation (the same volume as medium/min.) and agitation (190 rotations/min.). The composition of the culture media are shown in the following table.

| | | |
|---|---|---|
| I) | glucose | 15 kg |
| | tartaric acid | 800 g |
| | $NH_4$-tartarate | 800 g |
| | $(NH_4)_2$-$HPO_4$ | 120 g |
| | $K_2CO_3$ | 120 g |
| | $MgCO_3$ | 80 g |

| | | |
|---|---|---|
| | $ZnSO_4 \cdot 7H_2O$ | 14 g |
| | $FeSO_4 \cdot 7H_2O$ | 14 g |
| | $H_2O$ | 300 l |

adjusted to pH 4.0 by HCl

| | | |
|---|---|---|
| II) | glucose | 15 kg |
| | $NH_4NO_3$ | 900 g |
| | $(NH_4)_2HPO_4$ | 120 g |
| | $K_2CO_3$ | 120 g |
| | $MgCO_3$ | 80 g |
| | $ZnSO_4 \cdot 7H_2O$ | 14 g |
| | $FeSO_4 \cdot 7H_2O$ | 14 g |
| | $H_2O$ | 300 l |

adjusted to pH 4.0 by HCl

### 2) Isolation and Purification.

The cultured medium was filtered and a sufficient amount of activated carbon (2 kg per 300 l) was added to the filtrate while stirring. It was set aside overnight and the carbon separated by filtration, washed with water, and eluated with methanol containing about 0.7% ammonia seven times. Evaporating off the solvent below 55°, the residue was dissolved with aqueous sodium bicarbonate and extracted with ether. Then the aqueous layer was acidified with dil. sulfuric acid and again extracted with ether. The ether layer was extracted with mono-potasium phosphate solution, and the latter was acidified, and extracted with ethylacetate. On ovaparating the solvent, the acidic substance crystallized out as prisms or plates (m.p. 150-158° with decomposition). After recrystallization from hot water and then ethanol-ligroin three times, the melting point rose to 164-5° (decomp.). Yield: 3.0 g from 300 l cultured medium. This acids is easily soluble in methanol, ethanol and acetic acid, fairly soluble in water, ether and ethylacetate, but insoluble in chloroform, benzene, toluene, petroleum ether and ligroin.

The analytical data agreed with 5-hydroxymethyl-furan-2-carboxylic acid. *Anal.* Calcd. for $C_6H_6O_4$: C, 50.70: H, 4.23. Found: C, 50.66; H, 4.65. The mixed m.p. with synthetic compound was not depressed (164-5° with decomp.). Moreover, its ultraviolet spectrum showed the maximum at 250 m$\mu$ coinciding with that of Turner et al.[3], and its infrared spectrum of a nujol mull and was completely identical with the synthetic compound as illustrated in Fig. 1, which showed the presence of a hydroxyl group (about 3260cm$^{-1}$), a carboxyl group (1650 and 1300 cm$^{-1}$), and a conjugated double

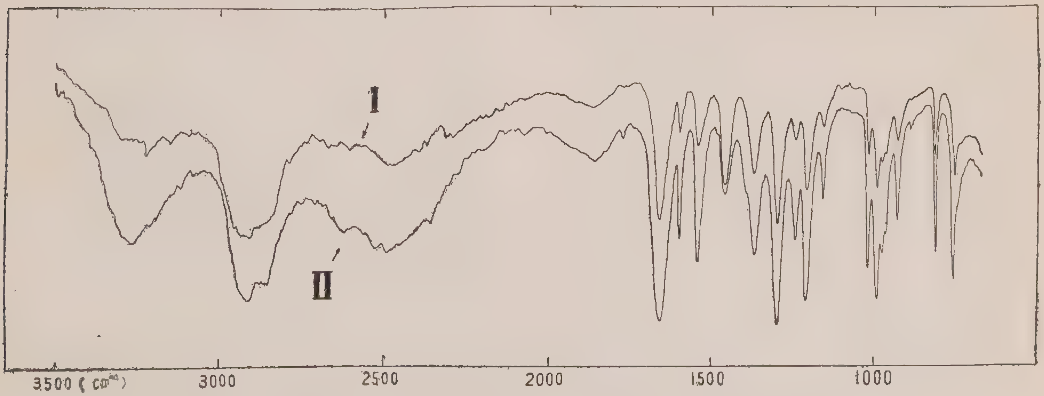

Fig. 1. Infrared spectrum of 5-hydroxymethyl-furan-2-carboxylic acid.

I. Synthetic compound. II. Isolated compound from fungus.

bond (1600 and 1540cm$^{-1}$) which may be due to a substituted furan linkage.

The authors wish to express their sincere thanks to Prof. Y. Sumiki for his kind guidance in performing this study.

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## 14. *Studies on Fungisporin. Part 2.*

By Kohei MIYAO

Fungisporin was subjected to acid and alkali hydrolysis and converted to amino acids. These amino acids were identified to be the two amino acids, L-phenylalanine and L-valine. From the results of the quantitative determination of each amino acid and infrared spectrum of the fungisporin, it was revealed that the fungisporin is a polypeptide composed of the same moles of L-phenylalanine and L-valine.

In the previous paper[1], it was reported that fungisporin, isolated from spores of several species of *Penicillium* and *Aspergillus*, was assumed to be a high molecular substance somewhat similar to a simple protein with respect to solubility, infrared spectrum, and the formation of the primary amines as its degradation products by heating with conc. hydrochloric acid. The present paper deals with the constituents of the acid and alkali hydrolyzates of the fungisporin as well as its molecular structure.

From the degradation product by acid hydrolysis, two amino acids were separated through their Cu-salts and were identified as DL-phenylalanine and DL-valine by the mixed melting points with the authentic specimens and from elementary analyses, indicating that racemization occurred during hydrolysis.

Moreover, paper chromatography of the acid hydrolyzate of fungisporin showed only two spots corresponding to phenylalanine and valine, and no other spots appeared. Furthermore, no other amino acid was detected by various qualitative analyses and paper chromatography of the alkali hydrolyzate, in an attempt to resolve the question of the presence of tryptophane which was unstable in the acid hydrolysis.

The experimental formula presented in the previous paper for the fungisporin should be corrected according to recent analytical data and from results described above to the formula, $(C_{14}H_{18}O_2N_2)_x$

The result of the quantitative determination of $\alpha$-amino nitrogen in the hydrolyzate showed that all the nitrogen of fungisporin was $\alpha$-amino nitrogen. To determine each amino acids component of fungisporin, the method of A.H. Gordon, A.J.P. Martin and L.M. Synge[2] was applied, and it was proven that fungisporin was composed of the same number of moles of these two amino acids and had no other residue. The above formula was completely agreed with not only this result, but also that of the derived by connecting same mole of valine and phenylalanine through simple amide linkage to give polypeptide having negligible end groups.

As to the optical configuration of the component amino acids, it was assumed to be the L-form because of the agreement of the values obtained on comparative bioassays of the hydrolyzate of fungisporin and a control which was subjected to the same procedure for the pure L-form amino acids.

The infrared absorption spectrum of a nujol mull of fungisporin is shown in Fig. 1.

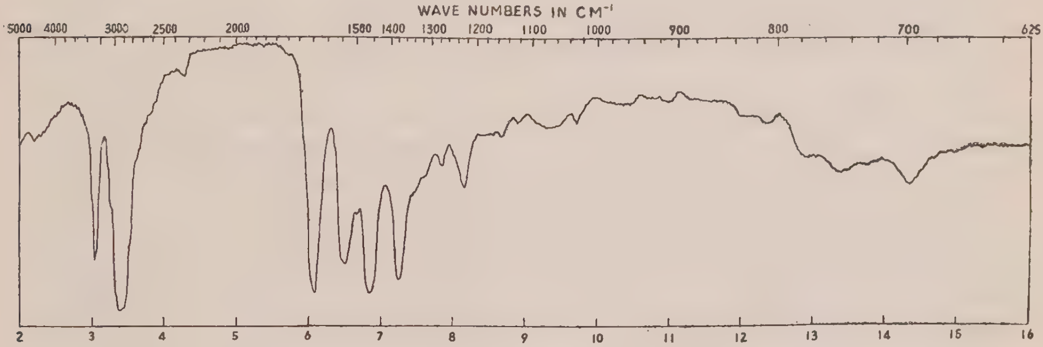

Fig. 1.

The presence of the bands at 3300, 3100, 1640, 1540, 1220 and 700cm$^{-1}$ shows definitely that fungisporin is the linear polypeptide type compound and not the diketopiperazine type.

The next step in the study of the structure of this peptide will involve partial hydrolysis in order to determine the sequence of the amino acids, and to determine its molecular weight.

These are being undertaken at present. It is interesting to note the fact that this fungisporin sublimes, despite its being a true peptide and not diketopiperazine type.

Further research of strains containing fungisporin is going on, and the species which have been found to contain the fungisporin at present are the following (concluding the species reported in the previous paper):

*Penicillum: notatum, chrysogenum, citrinum, claviform, purpurogenum, herquei, frequentaus, roseo-purpureum, brevicaule.*

*Aspergillus: niger, vercicolor.*

## *Experimental*

### 1. Identification of phenylalanine and valine as the component amino acids.

As described in the previous paper from the degradation product of fungisporin by heating with conc. HCl at 200° for several hrs., a primary amine $C_8H_{13}O_2N$, m.p. 249° (decomp) was obtained by recrystallization. This crystal gave a positive ninhydrin reaction and its benzenesulfonyl derivative. The above product was titrated potentiometrically by N/20 NaOH and N/20 HCl, using the Beckmann pH meter G-type. Its titration curve showed the buffer action in both acidic and basic sides, and the addition of formalin made it acidic. These phenomena indicate the substance to be a mixture of amino acids.

150 mg of this substance was boiled with freshly prepared copper hydroxide and filtered while hot. On cooling, the sparlingly soluble Cu-salt precipitated as light blue leaflets and filtered off. The precipitate was followed by washing with water, recrystallization from hot water three times, and dried (96 mg) (1). The filtrate was evaporated to dryness, and extracted with methanol and then water. Concentrating these extracts, the blue leaflets were preciptated by the addition of ethanol from the each extract. These preciptates were, recrystallized and dried: methanol soluble (11) (6 mg), water soluble (111) (63 mg).

The Cu-salts were analyzed and freed from the copper by treating with hydrogen sulfide but the methanol soluble Cu-salt quantity was too little and was completely used up by analysis.

Analytical figures of (1) agree with the Cu-salt of phenylalanine. Data of (11) and (111), also agreed with that of Cu-salt of valine.

Calcd. for $(C_9H_{10}O_2N)_2Cu$: C, 55.21 H, 5.12 N, 7.21 Ash, 20.02
Found. C, 54.95 H, 4.48 N, 7.20 Ash, 20.11
Calcd. for $(C_5H_{10}O_2N)_2Cu$: C, 40.65 H, 6.77 N, 9.40 Ash, 26.9
Found. of (11) C, 40.94 H, 6.06 N, 9.10 Ash, 26.7
Found. of (111) C, 41.02 H, 6.58 N, 9.32 Ash, 26.8

#### a) Identification of DL-phenylalanine.

The free amino acid obtained from the slightly soluble Cu-salt was given the experimental formula $C_9H_{12}O_2N$, from the analytical data which corresponded to that of phenylalanine. The benzoyl derivative of this amino acid, followed by recrystallization from water and ethanol showed m.p. 186°. This melting point was not depressed by an admixture of benzoyl DL-phenalalanine (m.p. 187°). Also the m.p. of the toluenesulfonyl derivative of this amino acid was 134° and was not depressed by mixing with the corresponding compound of DL-phenylalanine. Calcd. for $C_9H_{11}O_2N$: C, 65.5 H, 6.67 N, 8.49. Found. C, 65.21 H, 6.28 N, 8.46.

#### b) Identification of DL-valine.

The analytical figures agreed with that of valine. Calcd. for $C_5H_{11}O_2N$: C, 51.26 H, 9.46 N, 11.95. Found. C, 51.47 H, 9.87 N, 11.24.

The mixed melting point of this benzoyl derivative (m.p. 132°) with corresponding derivative of DL-valine was not depressed. The methanol soluble Cu-salt was assumed to be a salt of optical active valine by its solubility and analytical data.

**c) Identification by paper chromatography.**

Paper chromotography was applied directly to the acid hydrolyzate emplying the following solvents: a mixture of butanol-acetic acid-water (4 : 1 : 5), or liq. phenol satud. with 0.3 % $NH_3$ water, or a mixture of 2,4,6-collidine-2,6-lutidine-water (1:1:1). The result was that no other amino acids were found than the two mentioned above. In alkali hydrolysis, fungisporin showed extrordinary resistance to the hydrolysis so that a considerably amount of it remained unchanged in the hydrolyzate with 25% barium hydroxide soln. at 150–155° for 6 days. The presence of tryptophane was not detected when this hydrolyzate was tested by the following methods: paper chromatography, Hopkins-Cole reaction,[3] Neubauer-Rhode reaction,[4] Folin-Denis reaction[5] etc.

## II. Experimental formula for fungisporin.

Repeating the elemental analysis of fungisporin gave the corrected analytical data and resulted in the establishment of new experimental formula, ($C_{14}H_{18}O_2N_2$).

Analytical data of fungisporin.

| C% | H% | N% | |
|---|---|---|---|
| 68.38 | 7.33 | 11.38 | Found. (sample purified by sublimation 2 times) |
| 68.30 | 7.29 | 11.33 | Found. (sample purified by sublimation 3 times) |
| 68.29 | 7.37 | 11.35 | Calcd. for $C_{14}H_{18}O_2N_2$ |

This formula agrees with the figure of the dehydrated condensation product of phenylalanine and valine.

$$\overset{\text{fungisporin}}{(C_{14}H_{18}O_2N_2)x} = \overset{\text{phenylalanine}}{xC_9H_{11}O_2N} + \overset{\text{Valine}}{xC_5H_{11}O_2N} - 2\,xH_2O$$

## III. Quantitative analysis of the component amino acids.

Since it was found out that fungisporin could be hydrolyzed producing almost no humin material below 140°, and that at the temperature below 110° it scarcely hydrolyzed with 36 % hydrochloric acid, the hydrolysis was carried out at 140–130° for 4–7 days in many cases. Preparation of sample: 49.16mg of fungisporin was hydrolyzed with 10cc of 36% HCl at 140–130° for 6 days followed by evaporation to dryness, dissolved in water, filtered to remove traces of undissolved residue and filled up to 10cc with water.

**a) *α*-amino nitrogen determination.**

0.5cc of above solution was applied to the determination of *α*-amino nitrogen by Van Slyke method and found to be 0.1346 mg (corresponding to 96.2% theoretical value, calculated as $N/C_{14}H_{18}O_2N_2+2H_2O$). This value indicated that all nitrogen in fungisporin molecule was *α*-amino nitrogens.

**b) Determination of component amino acids.**

( 1 ) By the method of A.H. Gordon, A.J.P. Martin, and L.H. Synge.

Satisfactory results were obtained by this method, using Mallinckrodt silicic acid for the control test. 5cc of the above solution was evaporated to dryness and acetylated according to the above mentioned method. This acetylated hydrolyzate was chromatographed and each of the separated fractions was titrated as described in the papers of the above mentioned authors. 5 g of silicic acid (Mallinckrodt analytical reagent) were colored by adding 3 ml 0.1 % aq. methyl orange soln. to it, and then suspended in chloroform containing 3 % butanol. This was placed into a chromatogram tube having an internal diameter 1 cm. The following results were obtained:

phenylalanine: 0.0785mM (12.95mg) 96.5% Calcd. as fungisporin took 2mole of $H_2O$
valine : 0.0777mM ( 9.07mg) 95.2% per each dipeptides by hydrolysis

( 2 ) By paper chromatography.

Amino acids of the hydrolyzate described were also determined by paper chromatography, employed the method of maximum color density.[6] Briefly, the procedures were as follows Paper: Toyo filter paper No. 51 (2 × 40 cm) (was satisfactory). Solvent: butanol-HAc-water (4 : 1 : 5) (upper layer). 2.5 l of each of the standard amino acid solutions of various concentrations, 1 mM/l, 2 mM/l, 3 mM/l, 4 mM/l, 5 mM/l, 6 mM/l, 7 mM/l, 7mM/l, 9mM/l, 10mM/l, were spoted on a strip and chromatographed, followed by developing the color with ninhydrin soln. Maximum color densities of the spots of these chromatograms were measured in percent transmission to blank paper by the densitometer, which was made in our laboratory for paper chromatography and had a slit of 2 mm × 6 mm and filter of 570mμ. Standard curves, which were prepared by plotting the max. density of each spot against its concentration in the semi-logarithmic paper, were straight. The concentrations of amino acids to be tested were determined from the max. color densities of their spots by comparing them with the standard curve of the corresponding amino acid. From this experiment the following results were obtained. (average)

phenylalanine: 28.2 mg in this hydrolyzate. 92 %
valine : 20.8 mg ,, ,, ,, 95.5 %
(recovery was calcd. from the fact that fungisporin takes 2 moles of water per dipeptide by hydrolysis.)

c) **Bioassay.**[7]

As it is almost impossible to carry out the hydrolysis without any racemization, I compared the results of bioassays applied to fungisporin hydrolyzate to a mixture of the same number of moles of L-phenylalanine and L-valine which was heated with HCl under the same conditions as the hydrolysis in order to determine the optical configuration of the component amino acids of fungisporin.

21.45 mg of fungisporin and a mixture of 12.38mg L-phenylalanine and 9.08mg L-valine were hydrolyzed with 36% HCl at 140–130° for 6 days. Results of bioassays, using *Lactobacillus arabinosus*, were as follows.

| | | | | |
|---|---|---|---|---|
| fungisporin: | L-phenylalanine | 7.36 mg | in 10cc hydrolyzate | 51.2 % |
| | L-valine | 6.12 mg | ,, ,, ,, | 59.6 % |
| control : | L-phenylalanine | 6.6 mg | ,, ,, ,, | 53.2 % |
| | L-valine | 5.5 mg | ,, ,, ,, | 60.1 % |

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Seiyaku, Ltd. for the help I received in the preparation of samples; to Dr. T. Shimanouchi and Mr. T. Miyazawa of Mizushima Laboratory, Department of Chemistry, University of Tokyo, for the infrared analysis and its interpretation; to Mr. S. Abe of Sakaguchi Laboratory, Department of Agricultural Chemistry, University of Tokyo, for his contribution of strains and to Mr. G. Tamura and Mr. Y. Suzuki of Sakaguchi Laboratory for their kind guidance of bioassay; The author is also grateful to Mr. J. Hattori and Mr. K. Higuchi for their cooperation in carrying out these experiments.

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## COMMUNICATIONS

# 15. *On a New Water-Soluble Ergot Alkaloid, Triseclavine*

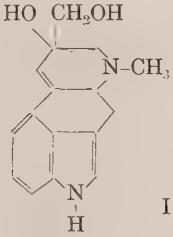
Matazô ABE, Saburô YAMATODANI, Tôgo YAMANO and Mitsugi KUSUMOTO

Last year Stoll et al.[1),2)] reported that they isolated a new alkaloid penniclavine, besides agroclavine and elymoclavine, from the saprophytic culture of the ergot fungus parasitic on African *Pennisetum typhoideum* Rich. and presented the formula I as its chemical structure.

Recently, we have found that some ergot fungi produce two unknown water-soluble alkaloids " A " and " B " both in nature and in saprophytic culture. We have succeeded in isolating " A " and " B " from the saprophytic culture of the *Elymus*-type ergot fungus parasitic on Japanese *Elymus mollis* Trin., applying starch-column partition chromatography. " B " has been isolated in excellent yield, also, from the saprophytic culture of the *Agropyrum*-type ergot fungus parasitic on *Agropyrum semicostatum* Nees, *Trisetum bifidum* Ohwi, *Festuca rubra* L. etc. in Japan.

Both " A " and " B " give a blue color with concd. sulfuric acid, but a green color with van Urk's and Keller's reagents. The ultraviolet absorption spectra of " A " and " B " are in good agreement with those of lysergic acids and the peptide-type ergot alkaloids. " A " crystallizes from ether, ethyl acetate, acetone, methanol, or ethanol in plates or prisms; m.p. 219°C (dec.), $[\alpha]^{18}_{D}=+154^\circ$, $[\alpha]^{18}_{5461}=+202^\circ$ (c. 0.2 in ethanol), $[\alpha]^{18}_{D}=+150^\circ$, $[\alpha]^{18}_{5461}=+188^\circ$ (c. 0.2 in pyridine). *Anal.* Found: C, 71.22; H, 6.76; N, 10.08, Calcd. for $C_{16}H_{18}O_2N_2$: C, 71.09; H, 6.71; N, 10.36%. It is insoluble in petroleum ether, almost insoluble in benzene, sparingly soluble in chloroform, ether, and ethyl acetate, moderately soluble in acetone, methanol, and ethanol, but readily soluble in pyridine. It dissolves appreciably in water, to give an alkaline solution, and readily in dilute acids. " B " crystallizes from benzene, ether, ethyl acetate, chloroform, acetone, methanol, or ethanol in prisms; m.p. 223°C (dec.), $[\alpha]^{18}_{D}=+143^\circ$, $[\alpha]^{18}_{5461}=+164^\circ$ (c. 0.2 in ethanol), $[\alpha]^{18}_{D}=+137^\circ$, $[\alpha]^{18}_{5461}= =175^\circ$ (c. 0.2 in pyridine). *Anal.* Found: C, 75.82; H, 7.46; N, 10.82. Calcd. for $C_{16}H_{18}ON_2$: C, 75.55; H, 7.14; N, 11.02%. It is insoluble in petroleum ether, sparingly soluble in benzene and ether, moderately soluble in chloroform, ethyl acetate, and acetone, but readily soluble in methanol, ethanol, and pyridine. As in the case of " A ", its solution in methanol or ethanol exhibits a marked fluorescence. It is less soluble in water than " A ", but readily soluble in dilute acids.

" A " is identical in all respects with penniclavine, while " B " is undoubtedly a new alkaloid, for which we propose the name triseclavine. It is probable that penniclavine and triseclavine are produced through elymoclavine and agroclavine, respectively. Triseclavine seems to be represented by the formula II.



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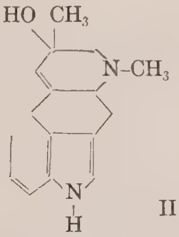

II

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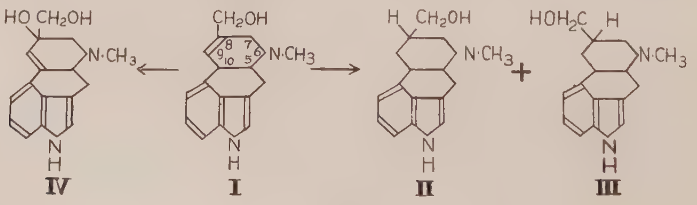
## 16. *The Structure of Ergot Alkaloid, Elymoclavine*

Saburô YAMATODANI and Matazô ABE

Previously, Abe et al.[1] presumed that elymoclavine had the formula I, in which the exact position of the easily reducible double bond must be between $C^8$ and $C^9$ or between $C^7$ and $C^8$. Our recent work has given a clue to the verification of the presumption. Abe et al. showed in the same report that elymoclavine yielded on catalytic hydrogenation a base, $C_{16}H_{20}ON_2$, m.p. 283° (dec.), $[\alpha]_D^{20}=-60°$ (ethanol), $[\alpha]_D^{20}=-92°$ (pyridine). We have since succeeded in obtaining from the mother liquor of the above-mentioned base an additional base in very small yield; leaflets from methanol, m.p. 188°, $[\alpha]_D^{15}=-40°$, $[\alpha]_{5461}^{15}=-59°$ (c. 0.11 in ethanol), $[\alpha]_D^{15}=-65°$, $[\alpha]_{5461}^{15}=-84°$ (c. 0.22 in pyridine). *Anal.* Found: C, 75.15; H, 7.73; N, 10.86. Calcd. for $C_{16}H_{20}ON_2$: C, 74.96; H, 7.86; N, 10.93%. At the same time, it was found that the previously obtained base and the newly obtained one are identical in crystal form, melting point, specific rotation, paper chromatographic behavior and infrared spectrum with dihydro-lysergol[2] (α-dihydrolysergol[3], formula II) and dihydro-isolysergol (I)[2] (β-dihydro-lysergol[3], formula III), respectively. This at once supports the presumption that elymoclavine has an alcohol group at $C^8$ and that the double bond in elymoclavine must be located between $C^8$ and $C^9$ or between $C^7$ and $C^8$. On the other hand, we have found that elymoclavine yields on oxidation with potassium bichromate in dilute sulfuric acid a basic substance; thick plates from methanol or acetone, m.p. 221° (dec.), $[\alpha]_D^{14}=+157°$, $[\alpha]_{5461}^{14}=+203°$ (c. 0.2 in ethanol), $[\alpha]_D^{14}=+153°$, $[\alpha]_{5461}^{14}=+192°$ (c. 0.2 in pyridine). *Anal.* Found: C, 71.04; H, 6.83; N, 10.23. Calcd. for $C_{16}H_{18}O_2N_2$: C, 71.09; H, 6.71; N, 10.36%. This substance is identical with pennicla-vine[4,5,6] (formula IV) not only in crystal form, melting point and specific rotation, but also in color reaction, chromatographic behavior, ultraviolet spectrum and infrared spectrum. The fact indicates that the double bond in elymoclavine is at the position between $C^8$ and $C^9$.

Moreover, it has been found that agroclavine also yields on the same oxidation a basic substance, $C_{16}H_{18}ON_2$, m.p. 225° (dec.), $[\alpha]_D^{16}=+145°$ (c. 0.2 in ethanol), $[\alpha]_D^{16}=+137°$ (c. 0.18 in pyridine), which is identical in all



respects with triseclavine[6]. This finding will support the view that the formula for triseclavine proposed by us in the preceding communication[6] would be correct.

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**Journal of the Agricultural Chemical Society of Japan.**

(Printed in Japanese)
Vol. 29, No. 1 (January, 1955)

1. K. Maekawa et al.: The Oxidation of Pepsin by Periodate.
2. Y. Mori: Studies on the Substances of Nucleic Acid System. I~II.
3. Y. Hatsuda et al.: Studies on the Metabolic Products of *Aspergillus versicolor.* III~IV.
4. S. Kataoka et al.: Raman Effect in Essential Oils II.
5. M. Takahashi et al.: Studies on the Production of a White Crystal "Glauconic Acid" and Red Pigment by *Penicillium purpurogenum.*
6. S. Fukuda: Studien über Sexualhormone. V.
7. K. Honda et al.: Enzymatic Study on Browning and Respiration of Potato. I.
8. T. Obara et al.: Studies on the Lecithin and cephalin in the Fat of Sawa-Millet Oil.
9. I. Yokotsuka et al.: Studies on Japanese Wine Film Yeasts. I. I Supplement.
10. K. Hayashida et al.: On the Photobleaching Chlorophyll.
11. H. Imai et al.: Studies on the Mold Prevention for Food Products. II.
12. A. Kuninaka: Studies on the Decomposition of Nucleic Acid by Microorganisms. II.
13. T. Harada et al.: An Unidentified Factor for Phenolsulphatase Production by Enterobacteriaceae.
14. T. Fukimbara et al.: The Action of Mold Amylase on Alcohol Fermentation. II.
15. N. Iguchi: Studies on Aspergilli. IX~X.
16. Y. Takeda et al.: Mycological Studies of Acetone-Butanol Fermenting Bacteria (KN-18) (*Clostridium kainantoi* nov. sp.).
17. H. Takehana et al.: Studies on Pectic Enzymes. III.
18. K. Matsushima: Studies on the Proteolytic Enzymes of Mold. VIII.

**Journal of the Agricultural Chemical Society of Japan.**

(Printed in Japanese)
Vol. 29, No. 2 (February, 1955)

19. S. Shinya: Studies on the Preparation of Cyclamen aldehyde and its Related Compounds. I~III.
20. K. Ueda: Studies on the Continuous Fermentation. IV~V.
21. T. Kondo et al.: Wood Extractives. III.
22. Y. Kudo et al.: One Constituent of the Leaves of *Daphniphyllum Macropodum* Mig.
23. Y. Minoda et al.: Studies on the Submerged Amylase Production. VI.
24. Y. Mori: Studies on the Substances of Nucluic Acid System III.
25. I. Yokotsuka et al.: Studies on Japanese Wine Film Yeasts. II~IV.
26. A Yoshida et al.: Studies on the Relationship between Nutrients and Metabolism of Body Fat. I.
27. Y. Inoue et al.: N-Glucosaminide I~II.
28. M. Tsujimura et al.: Studies on the Flavoring Substances of Green Tea. I.
29. I. Uritani et al.: Phytopathological Chemistry of Black-rotten Sweet Potato. XV~XVII.
30. T. Nakabayashi: Studies on Tannins of *Spirogyra arcta.* IV.
31. S. Omata et al.: On the Colours of Soy-sauce. III.
32. K. Kurasawa et al.: Sugar-dehydrogenase of Mold. II.

Letters to the Editor

- F. Yoshida: Crystallization of New Proteolytic Enzyme from *Aspergillus Saitoi*
- K. Izaki et al.: Degradation of L-Glutamic Acid in Bacterium.

(*Continued on inside back cover*)

(*Continued from outside back cover*)

**Journal of the Agricultural Chemical Society of Japan.**

(Printed in Japanese)
Vol. 29, No. 3 (March, 1955)

33. T. Suto: Azotobacter in Volcanic Ash Soil in Tohoku Distinct. I.
34. H. Okazaki: Studies on Amylases of Various Species of Mold. IX.
35. K. Tamari et al.: On the Biochemical Studies on the Blast Mould. II.
36. K. Tamari et al.: Studies on the Synthesis of 2-Isovaleryl-4-hydroxy-6-isobutyl-pyrone (2) and 3-[2-Methyl-butenyl (2)-on (4)]-hydroxy-6-[2-methyl-propenyl (2)]-pyrone (2) and on the Test of their Antibiotic Actions and Toxities.
37. Y. Hamamura et al.: Studies on the Redox Resin. I.
38. K. Yagi: Studies on the Components of the Bark of Rhamnus Japonica. XIII.
39. H. Imai et al.: Studies on the Mold Prevention for Food Products. III.
40. H. Kurasawa et al.: Sugar-Dehydrogenase of Mold. III.
41. H. Kuroiwa et al.: Non reducing Oligosaccharide-synthesizing Enzyme from Sucrose in *Aspergillus oryzae*. IV.
42. S. Omata et al.: On the Colour of Soy-Sauce. IV.
43. T. Tabuchi: Microbial Degradation of Nicotine and Nicotinic Acid. II~III.
44. S. Chikubu et al.: Studies on Thiamine of Cereals and Beans by the Photographic Method. IV.
45. M. Yatazawa: The Absorption by Plants of unseparated fission Products derived from H-Bomb desonated in Spring 1954 at Bikini-Atoll.
46. R. Sasaki et al.: Studies on the Determination of Vitamin A and Carotene in Butter.
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